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THE EFFECT OF WATER AND POTASSIUM CHLORIDE INGESTION ON THE LEVEL OF URINARY 17-KETOSTEROIDS IN THE RABBIT

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MUCH evidence indicates that adrenal cortical extracts and desoxycorticosterone acetate promote the excretion of ingested water and provide protection against lethal water intoxication in both normal and adrenalectomized animals (Gaunt, Birnie, and Eversole, 1949). Furthermore desoxycorticosterone has been found to be an effective stimulant for potassium excretion in the adrenalectomized animal. In view of these facts one might expect the normal experimental animal to respond to excessive quantities of potassium or water with an increased production of adrenal cortical hormones facilitating water or potassium excretion. McHenry *et al.* (1947) observed such a correlation between urine volume and the excretion of 17-ketosteroids in patients with carcinoma of the prostate as well as in normal subjects although thorough study of the effect of the administration of large quantities of water upon the level of urinary 17-ketosteroids was made in but one instance. On the other hand, Pincus (1943) reports no significant increase in the rate of 17-ketosteroid excretion during forced diuresis in one subject studied. Similarly Kimeldorf (1948) and De Koning *et al.* (1948) found no correlation between the fluctuations in daily urine volumes and the 17-ketosteroid excretions in normal rabbits. The present investigation was undertaken in order to determine possible alterations in the excretion of 17-ketosteroids as a result of prolonged diuresis or excretion of excessive quantities of potassium.

METHODS

Eight female New Zealand rabbits weighing from 2 to 3 kilograms were fed a stock ration of Rockland rabbit diet and water *ad libitum*. Two twenty-four hour normal urine samples were collected from each rabbit. Following this control period, 3 cc. of water

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per 100 gm. body weight were administered by stomach tube three times daily for two days. On the fifth and sixth days an equal volume of a one per cent solution of potassium chloride was given by stomach tube three times daily.

Each twenty-four hour urine sample was collected without preservative and analyzed the following day for chloride by the Volhard-Arnold method and for neutral 17-ketosteroids by a modification of the Engstrom and Mason (1943) method. The hydrolyzed urine samples were extracted with carbon tetrachloride which was washed, evaporated to dryness and the residues dissolved in an appropriate amount of absolute alcohol. The Zimmerman reaction was developed using 2.5 *N* KOH in absolute alcohol, and 2% m-dinitrobenzene and incubated at 37 degrees centigrade for 45 minutes. Duplicate standards of dehydroisoandrosterone[1] and duplicate blanks were run with each determination. Colorimetric analyses were performed on a Coleman spectrophotometer at 520 millimicrons (maximum absorption).

RESULTS

Forty-eight hour urine volumes for eight female rabbits appear with the total 17-ketosteroid and chloride content of each in table 1. During the

TABLE 1. RABBIT 48 HOUR 17-KETOSTEROID EXCRETION AS A FUNCTION OF WATER AND KCl INTAKE

Rabbit	Weight gm.	Control			Water			1% KCl		
		Urine volume cc.	Chloride m. eq.	17-KS* mg.	Urine volume cc.	Chloride m. eq.	17-KS mg.	Urine volume cc.	Chloride m. eq.	17-KS mg.
1	2912	353	17.4	4.47	595	19.2	8.23	525	79.7	6.16
2	2063	445	4.1	2.95	540	17.1	6.60	655	63.5	11.92
3	2434	312	21.2	6.16	600	16.4	6.91	550	88.0	6.42
4	2406	493	9.2	4.67	475	12.6	5.98	665	79.7	6.64
5	2434	415	15.0	4.16	661	14.8	7.15	500	73.4	5.40
6	2547	515	25.1	5.85	1020	14.0	10.39	1100	71.3	14.85
7	2688	215	10.0	3.58	513	17.8	5.24	410	62.7	3.51
8	2128	450	10.6	4.09	1170	11.2	11.79	1235	60.7	11.51
Averages		400	14.1	4.49	697	15.4	7.79	705	72.4	8.30

P_{2-1}† <.01; P_{3-1} <.05; P_{1-2} >.1‡

* 17-ketosteroids.
† Probability of a chance occurrence of the difference between the means, from Fisher's Table of T values.
‡ Difference not statistically significant.

forty-eight hour control period the average urine volume of 400 cc. contained 4.49 ± 1.01[2] mg. of 17-ketosteroids. The administration of water equivalent to 3 cc. per 100 gms. of body weight three times daily increased the average forty-eight hour urine volume to 697 cc. containing 7.79 ± 2.10 mg. of 17-ketosteroids. The increased excretion of steroid occurred to varying degrees in each of the eight animals studied. Statistical analysis of the difference of the means indicates this increase of 17-ketosteroid excretion to be highly significant.

When a 1% aqueous solution of potassium chloride was substituted for the water previously given, the average urine volume increased slightly to

[1] Dehydroisoandrosterone was generously supplied by Ciba Pharmaceutical Products Inc., Summit, New Jersey.

[2] Mean ± standard deviation.

705 cc. per forty-eight hours. A five fold increase in chloride excretion occurred in this period. The average increase of 58.3 m. eq. of chloride excreted over that of the control period provides a rough estimate of the m. eq. of potassium excreted during this forty-eight hour period. The average 17-ketosteroid content ofthis urine rose to 8.30 ± 3.68 mg. This moderate increase in 17-ketosteroid excretion over that observed when water was given alone is not statistically significant, however when compared with the average control value the difference of the means is again significant.

The correlation coefficient[3] for urine volume and 17-ketosteroid excretion was determined for the twenty-four urine specimens irrespective of previous treatment. A high degree of positive statistical correlation was found to exist between the two variables ($r = +.865$), indicating urinary 17-ketosteroids vary directly as the urine volume.

DISCUSSION

The variety of methods for the determination of urinary 17-ketosteroids and the individual modifications of each has established a relatively wide range of normal values. The 17-ketosteroid excretion for normal female rabbits reported in this paper are similar to those published by Davis *et al.* (1949). Other workers (Kimeldorf, 1948; De Koning *et al.*, 1948) using the Girard purification have reported normal rabbit 17-ketosteroid excretion at a somewhat lower figure.

Most of the past work concerning a possible correlation between urine volume and 17-ketosteroid excretion has been conducted on the relatively small variations observed in day to day urine volumes. In the human subject in which a water diuresis was evoked (Pincus, 1943), the shortness of its duration might explain the negative results obtained. It was found that the rabbits used in this experiment often did not respond to the diuresis of the first twenty-four hours with an increased excretion of 17-ketosteroids, but the second day's urine was found almost invariably to contain relatively large quantities of this material.

The clinical application of this determination as a diagnostic aid in such endocrine disturbances as Addison's disease, Cushing's syndrome, and hirsutism has received considerable attention. It is the belief of the authors that urine volume, relative to normal, should receive some consideration from the clinician in the evaluation of such tests.

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[3] $r = \dfrac{\Sigma\,(xy) - T_x T_y / N}{\sqrt{[\Sigma(x^2) - T_x^2/N]\,[\Sigma(y^2) - T_y^2/N]}}$ from Goulden, C. H.: *Methods of Statistical Analysis*, 1936, Burgess Publishing Company, Minneapolis, Minn.

SUMMARY

The urines of eight rabbits subjected to water diuresis for forty-eight hours were found to contain significantly greater quantities of 17-ketosteroids than control urines from the same animals. The ingestion of 180 mg. of potassium chloride per 100 gm. body weight as a 1% aqueous solution over a forty-eight hour period did not consistently cause a further increase in 17-ketosteroid excretion. A significant positive correlation between urine volume and 17-ketosteroid excretion was found to exist.

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SEROLOGIC BEHAVIOR OF CRYSTALLINE PREPARATIONS OF HYPOPHYSEAL GROWTH HORMONE[1]

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THE existence of the anterior hypophyseal growth hormone and the preparation of a protein from the anterior lobe of ox pituitary which had the biological characteristics of the growth hormone have been demonstrated in a series of papers (Evans, 1936; Li and Evans, 1944; Li, Evans and Simpson, 1945; Li, 1947 and Li and Moskowitz, 1949) found that the purified protein although not crystalline, behaved as a single component by criteria of diffusion, electrophoretic mobility, solubility and ultracentrifugation. By the latter method, the molecular weight was calculated to be 44,000. The isolation of a crystalline growth hormone was announced by Fishman, Wilhelmi and Russell (1947) and by Li, Evans and Simpson (1948) using similar techniques, the protein in each case having only growth-promoting qualities.

The antigenicity of certain hormonal proteins has been unequivocally demonstrated by means of the quantitative precipitin technique of Heidelberger and Kendall (1935). Such hormones as thyroglobulin and pituitary interstitial-cell-stimulating hormone were shown to behave in the quantitative precipitin test in a manner that clearly indicated the property of host-specificity in the case of the former (Stokinger and Heidelberger, 1937), and even of lobe specificity in the case of the latter (Chow, 1942).

Since the biological activity of the crystalline hypophyseal growth hormone was identified with a preparation homogeneous according to the four physical methods used for such determinations it was of interest to study the fifth criterion of homogeneity, i.e., immunochemical, on at least two crystalline preparations. Studies on the antigenic activity of two such preparations form the basis of this communication.

EXPERIMENTAL

Preparation of Antigens. The crystalline hormonal protein was dissolved in phosphate buffer pH 8.4 to give the desired concentration. Further dilutions were made in the buffer described below. Two lots of hormone were used in this work.

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To prove that the specificity of the precipitation tests and anaphylactic sensitizations was a function of the purified hormonal protein, and was not due to serum or tissue components from which the hormone was inseparable by all of the physical methods used previously in studying the protein, and for the presence of which no evidence exists in the physical studies conducted to date, a series of control antigens was used including: (1) ox serum, (2) 0.85 per cent sodium chloride extracts of whole ox pituitary tissue and (3) a supernatant fluid obtained during the course of purifying the hormone which also contained most of the possible contaminating substances.

These control antigens were used in the precipitation test both in the initial reaction, as well as in the tests for antibody excess in the homologous system, and as shocking antigens in the anaphylaxis experiments. In the precipitation reaction, increasing amounts of control antigens were added to the rabbit anti-hormone serum as in the homologous series, and in addition, aliquots of the supernatant fluids from the homologous system were tested for excess antibody in one case by the addition of more homologous (hormone) antigen and in the other case by the addition of the control antigens.

Quantitative Precipitin Tests. The technique of Heidelberger and Kendall (1935) was followed, with the additional procedure of adjusting all reagents to pH 8.4. Diluent for antigen dilutions was composed of tris-hydroxymethylaminomethane made to pH 8.4 according to the procedure of Gomori (1946). 2.43 gms. tris-hydroxymethylaminomethane were added to 100 ml. distilled water. To 25 ml. of this solution were added 12.5 ml. N/10 HCl. The total volume was brought to 100 ml. with distilled water. All precipitates were washed three times with chilled 0.85 per cent sodium chloride which did not contain the amino methane.

Preparation of rabbit antisera. Rabbits weighing about 5 lbs. were injected subcutaneously with varying amounts of hormone. One group of animals received on each of four successive days 1 mgm. of hormone; a second group received 1 mgm. on each of four successive days per week for three weeks; a third group received 5 mgms. per day four times weekly for one week; and a fourth received 5 mgms. per day on four successive days for each of 3 weeks. After a rest period the length of which depended on trial titrations of the sera by end-point titer method using constant amounts of antiserum, the animals were bled (4 to 6 weeks following last injection of hormone) and the serum frozen until needed.

Sensitization of guinea pigs. Groups of five guinea pigs, each animal weighing about 350 grms., were injected subcutaneously. One group received 1 mgm., and a second group received 5 mgms. on each of four suc-

cessive days. After six weeks the animals were tested for anaphylactic sensitization.

Passive sensitization of guinea pigs was produced by the intracardial injection of 4 ml. of undiluted rabbit serum from one of the animals in group four above. Forty-eight hours later the animals were given the "shock" dose of antigen, also intracardially. Control animals in this group received the same sensitizing doses of hormone, but were "shocked" with 2 ml. ox serum, or with 10 mgms. pituitary tissue antigen controls. Intravenous injections of the shock dose were also tried in each case.

RESULTS

Table 1 presents the data obtained in the active anaphylactic sensitization experiments. Large amounts of antigen were needed for the production

TABLE 1. ANAPHYLACTIC SENSITIZATION OF GUINEA PIGS WITH VARYING AMOUNTS OF ANTERIOR HYPOPHYSEAL GROWTH HORMONE

Number guinea pigs used	Sensitizing dose of hormone (mgms.)	Shock dose of hormone (mgms.)	Results				
			Deaths	Severe	Moderate	Slight	No reaction
1	4*	1					1
3	4	5	1	1	1		
1	4	7.5				1	
3	4	10.0	3				
1	20	1			1		
4	20	5	2			2	
3	20	9	3				
3	20	2 ml. ox serum (control No. 1)					3
3	20	10 mgms. control No. 2					3
3	20	10 mgs. control No. 3					3

* Much smaller doses had previously been tried with no sensitization resulting therefrom.

of the shock reaction. However, a marked improvement in degree of sensitization was not noted when the sensitizing dose was increased from a total of 4 mgms. to 20 mgms., since 6 out of 8 animals in each case responded with moderate or more severe forms of shock. The amount of antigen between the range of 4 to 20 mgms. does not appear to affect markedly the degree of sensitization to shock doses of antigen ranging from 1 to 10 mgms.

The animals passively sensitized with rabbit anti-hormone serum responded to a dose of 10 mgms. hormone only with severe but non-fatal anaphylactic shock. Recovery of both animals ensued in 15 minutes after the onset of symptoms.

In the guinea pigs which had been sensitized with the purified hormone preparations no reactions were elicited upon injection of the control anti-

gens indicating that the antigen preparation did not sensitize the guinea pigs by virtue of those particular serum or tissue components present in the control preparations. This did not however remove the possibility of other unknown contaminating antigens. It is, therefore, believed on the basis of these experiments that the growth hormone of the anterior hypophysis is either a relatively weak antigenic substance or, more likely, that the preparation used for this section of the study contained an antigenic contaminant(s).

TABLE 2. ADDITION OF INCREASING AMOUNTS OF HYPOPHYSEAL GROWTH HORMONE TO POOLED RABBIT ANTI-HORMONE SERUM (20 ML. SERUM, UNDILUTED)

Antigen N added (mgms.)	Total N pptd. (mgms.)	Antibody N pptd. (mgms.)	Ab-N / Ant-N	Supernatant
0.007	0.112	0.105	15.0	Excess antibody
0.015	0.134	0.119	7.9	Excess antibody
0.080	0.220	0.140	1.7	Excess antibody
0.140	0.296	0.156	1.1	Excess antibody
0.155	0.320	0.165	1.0	No excess antibody; no excess antigen
0.170	0.358			Excess antigen
0.200	0.373			Excess antigen
0.232	0.423			Excess antigen
0.775	0.450			Excess antigen
1.55	0.340			Excess antigen
3.10	0.170			Excess antigen

The results of precipitin tests on the first lot of hormone indicated that only sera from the animals receiving the four daily injections of 5 mgms. for three weeks contained enough precipitin even to attempt a quantitative precipitin test. These sera contained so little antibody to the hormone preparation that 20 ml. of undiluted serum (pooled) were used for each amount of antigen tested. Table 2 shows the effect of increasing amounts of hormone upon the pooled rabbit anti-hormone serum. The amounts of antibody present in the pooled sera were again more indicative that a contaminating substance was responsible for the precipitation reaction, although no precipitation occurred with the "anti-hormone" serum when the control antigen preparations were employed. Tests on aliquots of the supernatant fluids from the homologous systems for excess antibody were also performed with the control antigens and although excess antibody was demonstrated with 0.005 mgms. hormone "antigen nitrogen," tests with varying amounts of the control extracts and ox serum were negative. The fact that the amount of nitrogen precipitated continued to increase until five times as much antigen had been added as first produced antigen-excess in the supernatant indicated (1) that at least two antigens were present in this preparation of the crystalline hormone, (2) that the hormone was the "contaminating" component of another antigen-antibody

system or (3) the hormone was aggregating in serum or the hormone was combining with serum fractions other than "antibody."

Since tests directed to answering points (2) and (3) above were completely negative, it was concluded tentatively that a non-antigenic hormone was contamined by an antigenic substance. Considering the molecular weight of the hormone as 44,000, it will be observed that the first two ratios in table 2 are similar to those obtained for a protein such as egg albumin of similar weight.

An attempt was therefore made with a second preparation of crystalline hormone protein to study its antigenicity. This time the hormone was used both alone and in the form of an alum precipitate. One ml. of sterile 1 per cent alum solution was used per 100 mgms. hormone. The same regime of injections as previously described was used.

No evidence of antibody formation was obtained in any of the animals, although longer periods of sensitization and rest were tried along with greater amounts of hormone. The first results can be ascribed to at least two substances, but as yet it is not possible to state whether the hormone was identical with the first substance reacting.

CONCLUSION

One preparation of purified hypophyseal growth hormone homogeneous by several methods and crystalline in form did not induce the appearance of precipitating antibodies in the rabbit or anaphylactic sensitization in the guinea pig, after an intensive series of hormone injections. The response of guinea pigs and rabbits to an earlier preparation of hormone is ascribed to a contaminating substance, which in itself was only weakly antigenic.

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THE INFLUENCE OF ADRENAL CORTEX AND THYROID ON THE LOSS OF NITROGEN IN URINE AFTER EXPERIMENTAL BURNS[1]

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FOLLOWING experimental burns an increase in the urinary excretion of nitrogen takes place (Cuthbertson, 1930). The amount of nitrogen excreted has been shown to be affected by certain factors such as the degree of burning (Cuthbertson, 1930; Clark, Peters and Rossiter, 1945) and the nutritional state of the animals (Croft and Peters, 1945; Sellers and Best, 1947). Although it has been repeatedly demonstrated in normal animals that protein metabolism may be influenced by alterations in the functional state of the adrenal cortex (Long, Katzin and Fry, 1940; Ingle and Thorn, 1940; Ingle, 1941) and of the thyroid (Boothby and co-workers, 1925, and others), conclusive evidence that endocrine factors are responsible or even implicated in the disturbance of nitrogen metabolism after injury is lacking. In this communication it is shown that nitrogen loss after burning may usually be abolished or reversed by removal of the adrenals when the resistance of the adrenalectomized animals is increased by daily injection of desoxycorticosterone acetate.

It appears that the loss of nitrogen after burning is little affected by feeding moderate amounts of thyroid substance but after thyroidectomy loss of nitrogen is somewhat less than occurs in normal animals. This and other evidence presented suggests that the thyroid gland is not causally concerned with the changes in protein metabolism which occur after burning.

EXPERIMENTAL

1. *Effect of burning on metabolic rate*

Eight male rats of the Wistar strain weighing about 100 gm. were used in the first experiment. Daily estimations of metabolic rate were taken by measuring oxygen consumption directly (Ferguson and Sellers, 1949) at a temperature of 30°C. for a period of 12 to 15 days. A standard burn was then produced under ether anesthesia by immersing the backs of the animals in water at 85°C. for 30 seconds. After the burn, estimations of metabolic rate were made for a further period of 6 to 9 days.

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[1] The experiments reported in this paper form part of a project supported by the Defence Research Board of Canada.

2. *Effect of burning on nitrogen excretion (normal rats)*

Nine male and 8 female rats ranging from 122 to 147 and 140 to 160 gm. in weight respectively were fed a daily diet of 15 gm. of a powdered stock ration[2] which was placed in metal containers outside individual metabolism cages in such a manner that the rat could not enter the container or readily scatter the food. After the first 2 or 3 days of feeding little scattering occurred. The amount of food offered was determined by a period of preliminary feeding. After the amount of food offered daily was consumed completely on each of 3 or more days, the collection of urine was started. This was accomplished by placing large funnels under cages with bottoms of galvanized screening and allowing the urine to fall into flasks containing concentrated sulphuric acid. A pledget of glass wool was placed in the funnel to decrease the possibility of contaminating the sample by scattered food. The samples were collected at 48-hour intervals, and after repeatedly washing the funnels, were made up to 100 cc. and filtered. 1 cc. aliquots were taken for duplicate estimations of nitrogen using a micro-Kjeldahl method. After 12 days of collection (male group) and 6 days of collection (female group) all rats were subjected to the standard burning procedure described above. The amount of nitrogen excretion before the burn, expressed in mg. of nitrogen excreted per 24 hours, was compared with the average daily excretion for 6 days after burning. The rats were weighed every other day in all these experiments.

3. *Effect of burning on nitrogen excretion after adrenalectomy*

Studies of the effect of burning on nitrogen excretion after adrenalectomy were complicated by the fact that adrenalectomized rats died several hours after a burn severe enough to cause a significant increase in nitrogen excretion. Therefore the protective effect of desoxycorticosterone acetate (DCA) against a burn stress was utilized (You and Sellers, 1950). Male rats weighing about 150 gm. were adrenalectomized and given physiological saline in lieu of drinking water after the operation. About 5 days later water was substituted for the saline and 1 mg. of DCA[3] was injected subcutaneously into each rat daily until the end of the experiment. Urine collections were started 3 days after the commencement of DCA injections. After 4 to 6 days of collecting urine the rats were burned (85°C.; 30 sec.) and collections were continued for 6 days more. At the end of this period blood NPN was estimated using the micro-Kjeldahl method in 19 of the 22 animals studied. When DCA was withdrawn at the end of the experimental period all but two animals died within 3 to 17 days (av. 10 days), indicating the absence of functional accessory adrenal cortical tissue.

Five experimental groups of 5 adrenalectomized rats were studied. In the *first series* each rat was fed 18 gm. of food, and urine was collected 4 days before and 6 days after burning. In the *second series* the same scheme was followed with the exception that 15 gm. of food was consumed daily by each rat. In the *third, fourth and fifth* series adrenalectomized rats were studied concurrently with the same number of non-adrenalectomized control rats which received the same amount of food (15 gm./rat/day in series 3, and 16 gm./rat/day in series 4 and 5) and were burned at the same time and at the same temperature as the experimental groups. Urine was collected for 4 days prior to burning in series 3 and for 6 days prior to burning in series 4 and 5, and in all series was collected for 6 days after burning.

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[2] Fox Breeder Cubes, Master Feeds, containing 20% proteins.

[3] Percorten, Ciba.

4. *Effect of thyroid medication on urinary nitrogen excretion*

(1) *First thyroid series:* Five male rats weighing 138 to 154 gm. were offered 15 gm. of food daily. After 8 days of urine collection desiccated thyroid[4] was added to the food in a concentration of 0.1%. The same amount of food was offered to each rat daily for a period of 12 days. The amount of urinary nitrogen excreted per 24 hours after feeding thyroid was compared with that excreted before thyroid medication.

(2) *Second thyroid series:* Ten male rats ranging from 116 to 130 gm. in weight were offered 13 gm. of food daily. After 10 days of urine collection the same amount of food containing 0.3% desiccated thyroid was offered and urine collections were continued for 10 days. The amount of urinary nitrogen excreted per day was compared with that excreted before feeding thyroid.

5. *Effect of burning on nitrogen excretion in rats fed thyroid*

(1) *First series:* The 5 rats which were given 0.1% desiccated thyroid in 15 gm. of food (experiment 4) were burned after 12 days of this dietary regime. Feeding of thyroid and collection of urine were continued for 6 days after burning.

(2) *Second series:* Ten days after the commencement of thyroid (0.3% thyroid in 13 gm. laboratory stock ration) 10 rats in the second thyroid series of experiment 4 were subjected to a standard burn. Three collections of urine were made after the burn.

TABLE 1. EFFECT OF BURNING ON NITROGEN EXCRETION. NORMAL AND THYROIDECTOMIZED RATS

No. of rats	Sex	Treatment	Mean daily excretion of nitrogen (mg./rat/diem)		
			Before burn	After burn	Difference (±S.E.)
9	M	Normal	250.0	310.4	60.4±11.1
8	F	Normal	266.9	330.0	63.1± 9.1
8	F	Thyroidectomized	231.5	256.1	24.6± 4.0

6. *Effect of burning on nitrogen excretion in thyroidectomized rats*

Ten female rats weighing about 160 gm. which had been thyroidectomized 20 days previously were allowed 10 gm. of food daily. The same procedure used in experiment 2 was followed with the exception that urine was collected for 6 days before the burn. Results from only 8 rats are included in Table 1, as two rats were discarded during the experiment. One of these failed to survive and the nitrogen excretion of the other was extremely variable both before and after burning.

RESULTS

In the first experiment in which the effect of burning on the metabolic rate (oxygen consumption) was studied there was no appreciable difference between the values before and those after burning (Figure 1) although there may have been a slight temporary reduction in the 24 hours following the burn (Figure 1, lower graph). The colonic temperature was subnormal for several hours after burning.

[4] Parke, Davis and Company 0.3% iodine.

In experiment 2, in which two groups of male and female rats were burned using the standard method, in most animals a definite increase in urinary nitrogen occurred after the burn. The results are shown in Table 1. The daily variation of urinary excretion of nitrogen over a period of 10 to 12 days was relatively small.

In series 1 and 2 of experiment 3, in which 10 adrenalectomized rats were injected daily with DCA and then were burned, one died after the burn, none of the nine surviving showed an increase in nitrogen excretion, and

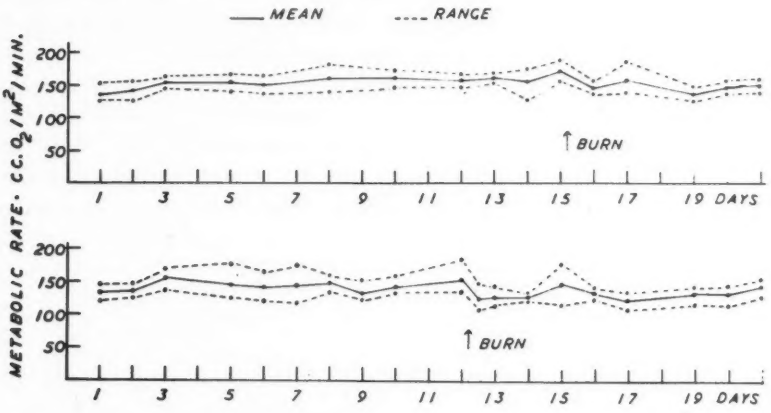

FIG. 1. Oxygen consumption of two groups of 4 rats before and after burning.

4 rats had a decreased excretion. Because of the altered reaction to burning by these adrenalectomized animals, normal control rats were burned concurrently in the remaining three series of experiments. It was found that of 15 adrenalectomized rats, 2 died after burning; of the 13 rats remaining a moderate increase in urinary nitrogen was found in 4 cases, and the others showed either no appreciable change or a decrease. Of the 15 normal control rats, decreased excretion was found in 2 cases, 10 rats exhibited a definite increase and 3 showed no appreciable changes. The blood NPN of the burned adrenalectomized rats (av. 49 mg.% in 19 rats) was within the range found in normal non-burned animals. In Table 3 the results obtained in this experiment are summarized. Of 22 adrenalectomized rats studied in the three series no appreciable change in nitrogen excretion occurred in about half (10), a marked reduction occurred in most (8) of the remaining cases while an increase was observed in 4 rats. During the first 48 hours following the burning, food consumption decreased similarly in both the control and adrenalectomized rats.

The effect on nitrogen excretion of administering two different dose

TABLE 2. EFFECT OF BURNING ON NITROGEN EXCRETION. RATS FED DESICCATED THYROID

No. of rats	Desiccated thyroid in food (%)	Mean daily excretion of nitrogen (mg./rat/diem)				
		Before thyroid feeding	During thyroid feeding	Difference (±S.E.)	After burn	Difference (±S.E.)
5	0.1	231.8	256.8	25.0±7.8	321.6	64.8±22.6
10	0.3	230.0	262.0	32.8±4.8	324.3	61.5± 6.9

levels of desiccated thyroid (experiment 4) is summarized in Table 2. A definite but slight increase in nitrogen excretion occurred in both cases, the higher dose level being associated with a greater increase.

When the rats fed thyroid were burned as in experiment 5, a further increase in nitrogen excretion was found (Table 2). The increase was similar in magnitude to that seen after a similar burn in normal rats.

In the sixth experiment, in which 8 fema'e thyroidectomized rats were studied, a significant increase in nitrogen loss occurred after the burn

TABLE 3. EFFECT OF BURNING ON NITROGEN EXCRETION. NORMAL CONTROLS AND DCA-INJECTED ADRENALECTOMIZED RATS

Change of N excretion after burn	Adrenalectomized group			Control group		
	No. of rats	Av.*	Range	No. of rats	Av.*	Range
Increase	4	40.8	28 to 55	10	53.6	24 to 100
No change	10	− 1.5	− 9 to 11	3	4.0	− 6 to 10
Decrease	8	−40.6	−70 to −25	2	−32.0	−34 to −30

* Average of difference of urinary nitrogen (mg./diem) before and after burning.

(Table 1). The increase was less than that usually seen in normal female rats.

DISCUSSION

The finding that there was no appreciable change in the metabolic rate in normal rats following a burn indicates that the changes in protein metabolism and other metabolic activities after burns are not produced by an alteration in the total rate of metabolism of the animal. Desiccated thyroid administered at a concentration of .3% in the food caused only a small increase in nitrogen excretion. As might be expected the steady increase in body weight seen in normal rats was reduced in these hyperthyroid animals. When they were burned the further increase in urinary nitrogen was of the same order of magnitude as that following burns in normal animals. The additive effects of thyroid feeding and of burning on the nitrogen excretion of rats suggest that a different mechanism may be involved in the two cases. That the thyroid gland is not causally related to

the increase in protein catabolism after burning is also illustrated by the fact that the excretion of urinary nitrogen still increased after the burn in the thyroidectomized rats. However, in thyroidectomized rats the nitrogen loss following the burn was less than that occurring in normal animals. This finding is in agreement with that of Peters (1948) in which he carried out a similar experiment using rats treated with thiouracil. It is possible that the difference in magnitude of loss between normals and thyroidectomized rats might be accounted for by the lower dietary intake of the latter animals.

Ingle (1949) has reported that in adrenalectomized rats no increase in nitrogen excretion takes place after burning, but that if a constant daily dose of 20 cc. of adrenal cortical extract is administered an increased excretion occurs. He concluded from this that the increased nitrogen excretion after burns or other injuries was not caused by over-activity of the gland but was dependent on the presence of cortical hormones. In our experience a burn severe enough to cause a definite increase in urinary nitrogen in normal rats, is nearly always fatal within a few hours in adrenalectomized rats. By utilizing a previous observation (You and Sellers, 1950) that DCA when administered for a period before a burn protected adrenalectomized rats against the lethal effects of burning, it was possible to produce a burn severe enough to increase nitrogen loss in the normal, and yet permit survival of the adrenalectomized animal. The lack of effect which DCA appears to have on protein metabolism in normal animals made it appear suitable for use in these studies. In normal rats DCA does not decrease the urinary nitrogen excretion after burning (Cuthbertson, Shaw, and Young, 1941; Sellers, You and Thomas, 1950).

In spite of using this method the results obtained with adrenalectomized rats are difficult to interpret. In previous nutritional studies the significance of the differences of nitrogen excretion between treated and untreated groups has been considered an acceptable measure of an effect. In this case if the results are treated in a similar way it is clear that removing the adrenal glands (and giving a maintenance dose of DCA) has produced a decrease in the urinary nitrogen excretion after burning as compared to normal controls.

More often than not there was either no increase at all or an absolute decrease in nitrogen excretion after burning. This would imply that the nitrogen loss after the injury was dependent on the action of the adrenal gland and would agree well with many previously formulated views on stress phenomena. If this were true, adrenalectomy should always produce this effect, but in our series there were several cases in which an *increase* of nitrogen loss did occur after burning. Presence of accessory cortical tissue, differences of food intake, or incomplete removal of the adrenals might ex-

plain the discrepancy, but in these experiments there is no reason to believe that these explanations are relevant.

Therefore it is necessary to postulate a mechanism which would account for the increases as well as the result more commonly seen, that is, no increase, or a decreased nitrogen excretion.

If it were true that nitrogen loss after burning depended on the presence of adrenal cortical hormones it would follow that the other factors influencing the loss of nitrogen (e.g. nutritional) must exert their effect in concert with the adrenal cortex. There is no satisfactory evidence that this is the case, as the role played by cortical steroids in intermediary metabolism is not well understood. The loss of nitrogen after injury differs from the typical "stress reaction" by frequently continuing unabated for many weeks. In 1946 Sellers and Parker showed that alleviating effectively the production of shock after experimental burns did not lower the increase in nitrogen excretion significantly. Of course, the production of traumatic shock presents a different problem in the study of stress phenomena, but one is tempted to assume that some close relationship may exist.

With the data available it must be admitted that, while the adrenal cortex is important in influencing this particular reaction to injury, other unknown factors play a part. What these may be remain to be discovered.

A further problem which remains unsolved is whether the loss of nitrogen should be invariably considered an undesirable side reaction or perhaps may serve some useful purpose. In the extreme case, loss of nitrogen from the body represents an uneconomic waste of tissue protein, but within limits many workers (Cuthbertson, 1932) have taken the view that it is a phenomenon inherent in the metabolic processes after injury, possibly connected with repair and should be regarded as a normal, even favorable reaction.

SUMMARY

After burning no appreciable change in metabolic rate was observed.

Administration of desiccated thyroid in the diet (0.1% and 0.3%) caused only a slight increase in urinary nitrogen excretion, much less than that seen after a severe burn.

When rats fed thyroid were burned, increased urinary nitrogen excretion took place of the same order of magnitude as that found after burning normal rats.

Thyroidectomized rats showed a slight but definite increase in urinary nitrogen after burning.

Nitrogen loss after burning was usually abolished or reversed in adrenalectomized rats when resistance of the adrenalectomized animals was raised by administration of DCA.

The results obtained indicate that thyroid function is not essential for change in protein metabolism after a burn while the function of adrenal cortex is an important but not an exclusive factor.

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EFFECT OF THYROIDECTOMY, ADRENALECTOMY AND BURNING ON THE URINARY NITROGEN EXCRETION OF THE RAT MAINTAINED IN A COLD ENVIRONMENT[1]

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AN INCREASED nitrogen excretion in the urine has been shown to occur after different forms of stress such as burning (Cuthbertson, 1930), traumatic injury (Cuthbertson, 1932) and exposure to a cold environment (Lusk, 1928). Studies of the mechanism involved have implicated certain nutritional (Croft and Peters, 1945; Sellers and Best, 1947; Chanutin and Ludwig, 1947) and endocrine factors (Ingle, 1949; Sellers, You and You, 1950). There is evidence that the thyroid gland is not causally related to the enhanced protein catabolism while the adrenal gland plays an important role (Sellers *et al.*, 1950).

In this paper experiments are described which indicate the relationship of the thyroid and the adrenal to the increased excretion of nitrogen which occurs in a cold environment. The effect on urinary nitrogen excretion when two separate conditions of stress, cold and burning, exist simultaneously is also reported.

In a cold environment both the thyroid and adrenal glands undergo hyperplastic changes and both are necessary for survival in the cold. Nevertheless it is shown that increased urinary nitrogen excretion in response to exposure to cold is not dependent on the presence of either the thyroid or the adrenal, as it occurs in the absence of either gland.

Lathe and Peters (1949) reported that in two experiments the effects of cold (17°C.) and burning on nitrogen loss were not additive although in one experiment carried out at a colder temperature (11°C.) nitrogen loss was increased by burning. The lack of additive effect in their first experiments suggested to these authors that some primary common pathway might be involved in the metabolic disturbance in the two conditions. In the experiments to be reported here, a lower environmental temperature (1.5°C.) was employed, and it was observed that loss of urinary nitrogen after burning was greater than when burning was carried out at room temperature.

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EXPERIMENTAL

1. *Urinary nitrogen loss after exposure to cold*

Ten male rats weighing from 120 to 137 gm. each were fed 15 gm. daily of commercial powdered laboratory ration,[2] and were allowed water ad libitum. Urine excreted was collected at 48-hour intervals (Sellers *et al.*, 1950) and nitrogen estimations were carried out on 1 cc. aliquots after dilution to a known volume. After 6 days at normal room temperature (23–27°C.) the rats, then weighing 130 to 156 gm., were transferred to a cold room (temperature 1.5 ± 1°C.). The same amount of food was allowed each rat for 4 days. After the first two collections of urine in the cold 30 gm. of food were offered (and consumed) daily and collections of urine were continued for an additional 4 days. The average excretion of nitrogen at room temperature (expressed as mg. of nitrogen per 24 hours) was compared with the average amount of nitrogen excreted in the cold.

2. *Effect of cold on urinary nitrogen in thyroidectomized rats*

Seven male rats weighing from 120 to 177 gm. (av. 155 gm.) which had been thyroidectomized 15 to 21 days previously, were fed 14 gm. of food daily. After collections of urine had been obtained for 6 days the animals were transferred to the cold room (1.5°C.) and urine was collected for a period of 24 hours. The same amount of food was offered as before but only two rats consumed the entire ration. The experiment was discontinued after 24 hours owing to the poor condition of the animals. The amount of nitrogen in the 24-hour specimen was compared with the 24-hour average obtained at room temperature.

3. *Effect of cold on excretion of urinary nitrogen in adrenalectomized rats*

Two female and 8 male rats weighing from 162 to 240 gm., which had been adrenalectomized 8 days previously, were given 1 mg. of desoxycorticosterone acetate (DCA)[3] in oil subcutaneously each day until the end of the experimental period. DCA was given in an attempt to prolong the survival time of the adrenalectomized rats so that a 24- or 48-hour urine specimen might be obtained in the cold environment. The rats were given physiological saline (0.9%) to drink ad libitum throughout the experiment. A period of preliminary feeding disclosed that the daily intake varied appreciably between rats, being 14 gm. (2 rats), 16 gm. (4 rats), and 20 gm. (4 rats). Collection of urine was started after 3 injections of DCA and was continued for the 4 days prior to exposure. The change in environmental temperature was effected by gradually lowering the temperature from 14°C. to 1.5°C. over a period of 7 hours. The period of survival permitted the collection of 24-hour specimens on 5 rats and 48-hour specimens on the remaining five. The average excretion of nitrogen for 24 hours before and 24 hours after exposure to cold was compared.

4. *Effect of burning on survival, and nitrogen excretion of rats exposed to cold*

First series: Seven of 10 rats survived the procedure outlined in (1). These were subjected to a standard burn under ether anesthesia by immersing the backs of all animals in water at 85°C. for 30 sec. The burning was carried out at room temperature and after some 3–4 hours (to permit recovery from the anesthetic) the rats were returned to the cold room.

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[2] Fox Breeder Cubes, Master Feeds, containing 20% proteins.

[3] Percorten, Ciba. Kindly supplied by the Ciba Company, Limited, Montreal.

Second series: Ten male rats of about 200 gm. in body weight were fed 25 gm. of food daily in the cold room. After 3 days of this regime collection of urine was started. Six days later a burn was produced in a manner similar to that described above except that a lower temperature (75°C.) was used. In this case collections were continued for 6 days after burning so that the average daily excretion of urinary nitrogen for 4 days before and 6 days after the burning could be compared.

Third series: Ten male rats weighing about 200 gm. were treated in a similar fashion except that 30 gm. of food was offered daily and that urine was collected for 4 days before and 6 days after the burning.

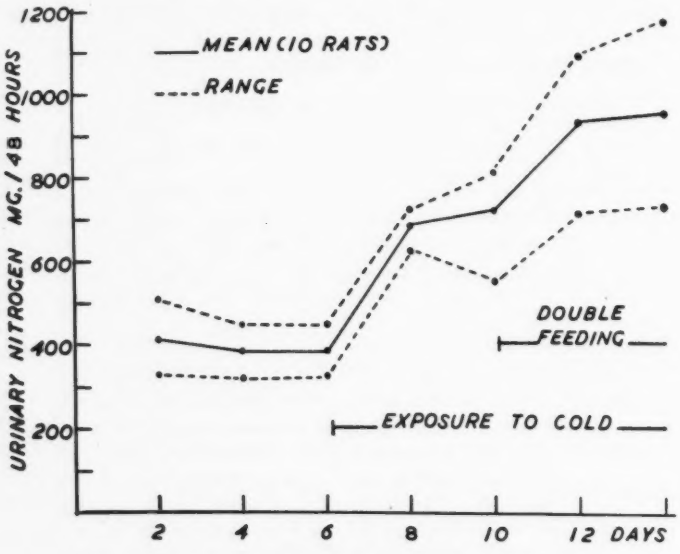

FIG. 1. Nitrogen excretion in the urine in 10 rats before and after exposure to a cold environment.

RESULTS

In the first experiment, in which the effect of cold on the nitrogen excretion of normal rats was studied, a great (77%) increase in urinary nitrogen excretion took place after exposure to a temperature of 1.5°C. Two animals died on the second day after exposure and one more on the fifth day of exposure. Presumably the limited amount of food offered could account for these deaths. The average body weight was reduced from 145 gm. to 119 gm. in 4 days. When the food intake was doubled a further marked increase in urinary nitrogen occurred (Fig. 1). In the succeeding four days the surviving rats gained about 11 gm. in body weight.

In the second and third experiments it was found that a marked increase in urinary nitrogen occurred when the thyroidectomized and adrenalec-

TABLE 1. EFFECT OF COLD (1.5°C.) ON NITROGEN EXCRETION OF THYROIDECTOMIZED AND ADRENALECTOMIZED RATS

No. of rats	Group	Mean nitrogen excretion (mg./diem/rat)		Difference ±S.E.
		Basal period	Cold period	
7	Thyroidectomized	236.7	329.4	92.7±20.3
10	Adrenalectomized	250.3	331.3	81.0±11.4

tomized rats were exposed to cold. The results are presented in Table 1. There was a reduction (av. 20 gm.) in body weight in all the thyroidectomized rats and a moderate, variable reduction in most of the adrenalectomized rats. Some retention of fluid and sodium, due to the effect of DCA may have occurred in the latter group.

In the first series of experiment 4 all the rats (burned at 85°C. for 30 sec.) died within several hours after re-exposure to cold. In the second and third series in which a total of 20 rats were subjected to burning at 75°C., 4 died before the termination of the experiment, so that data on only 16 rats are available. In most instances in both the latter series a further increase in nitrogen excretion took place above that due to cold alone (Table 2). The "P" value for the mean difference of nitrogen excretion for the combined groups before and after the burning is less than 0.01 ($t = 3.68$). Thus the effects of cold and burning on nitrogen loss were cumulative.

The body weight of all rats decreased during the first few days after exposure to cold but remained relatively constant (av. about 180 gm.) in the period during which urine was collected. After the burn there was a slight increase in body weight during the first two days but a moderate decrease during the last four days.

TABLE 2. EFFECT OF BURNING ON NITROGEN EXCRETION OF RATS IN COLD (1.5°C.)

No. of rats	Daily food intake gm.	Mean nitrogen excretion (mg./diem/rat)		Difference ±S.E.	"P" value*
		Before burn	After burn		
7	25	470.6	496.6	26.0±12.8	0.05–0.10
9	30	542.8	587.9	45.1±14.9	0.01–0.02

* When the results obtained from the two groups are combined the "P" value is less than 0.01.

DISCUSSION

The results of this investigation show quite definitely that an increased excretion of nitrogen in the urine occurs when rats are exposed to a temperature of 1.5°C. and that the increase is not dependent on the presence of either the adrenal or the thyroid glands. It may be concluded that the increased protein catabolism at this temperature may be affected by, but

is not primarily due to the action of the thyroid or the adrenal glands. The dispensability of the thyroid gland in the reaction is not unexpected since thyroid hyperplasia (Kenyon, 1933) and increase in "basal" metabolic rate (MR 30°) (Sellers and You, 1950) take place over a period of at least several days after rats are exposed to cold, while nitrogen excretion increases greatly within a matter of 24 hours. However, the demonstrable changes in the adrenal cortex in response to a cold stress occur within several hours (Sayers and Sayers, 1947). This, together with the importance of adrenal function in relation to the increased nitrogen excretion after burns (Ingle, 1949; Sellers, You and You, 1950) makes similar studies using a "cold" stress timely. The definite increase in nitrogen excretion in DCA-treated adrenalectomized rats in a cold environment indicates that unlike after burning, the adrenal gland is not primarily concerned, at least under the conditions of the experiments reported. Nevertheless there is good evidence that a relationship between thyroid and adrenal function, and metabolism in cold does exist. The presence of the thyroid and adrenal hormones, and perhaps also increased secretion of adrenal cortical principles may play a part in the full response seen in normal animals.

In this connection it is well to consider some of the effects of exposure to a temperature of 1.5°C. The metabolic rate (oxygen consumption is increased to more than twice its former value, food consumption rises to almost double, yet rate of growth is poor. There is ample evidence that such an exposure also produces a reaction affecting many tissues and metabolic functions (stress reaction). In young rats the series of phenomena often terminate fatally within a few days.

An increased nitrogen excretion is commonly associated with conditions in which metabolic rate is increased, such as after the administration of thyroid substance or of epinephrine. The loss of nitrogen appears to be linked in part with the caloric requirement. Though the metabolic rate may be raised considerably in heavy muscular exercise, in this condition the sparing action of carbohydrate and fat on body protein is well known. Whether the loss of nitrogen during exposure to cold might be influenced by varying the proportion of various dietary constituents, thus "sparing" protein, remains to be seen.

A degree of burning, which allows animals living in normal temperature to survive, is fatal to animals in an environment of 1.5°C. It has also been shown that severe cold greatly increases the susceptibility of animals to burn shock. In these experiments when a milder burn (at 75°C.) was produced most of the rats survived in the cold environment and studies on the combined effects of burning and cold were made possible. The increase in nitrogen loss after the burn appeared to be added to the increased loss due to cold. This is the more significant for when burning (at 73°C.) was carried

out using the same technique on animals kept at room temperature there was no appreciable increase in excretion of urinary nitrogen (Sellers and Best, 1947). Thus a relatively mild injury, which presumably would produce slight, if any, increase in nitrogen loss at room temperature, in the cold produced a loss as great as that of a more severe burn at room temperature, and this was superimposed on the loss which ordinarily occurs at 1.5°C. This suggests that relatively mild burning in addition to another condition of stress (cold) produces an effect greater than might be expected from simply adding together the effects of the two processes. If this is correct the total increase in nitrogen loss after burning and exposure to 1.5°C. would be made up of a part due to the combined stress, and a part due to increased metabolic rate. The results of the studies on thyroidectomized and adrenalectomized animals would support the view that different mechanisms are involved in the increased nitrogen loss after burning and after exposure to cold.

SUMMARY

In a cold environment (1.5°C.) rats excreted 77% more nitrogen in the urine than at normal room temperature (25° ± 2°). When the food intake in the cold was doubled the urinary nitrogen was increased further to about 2.3 times its original value. Growth as measured by increase in body weight was slow, however.

After exposure to cold a marked increase in urinary nitrogen occurred in both thyroidectomized and DCA-injected adrenalectomized rats.

The effects of a mild burn and of exposure to cold on urinary nitrogen excretion were cumulative and under the conditions of the experiments appeared to be greater than would be expected from the sum of the nitrogen losses due to the two stimuli acting independently.

The thesis that a different mechanism is involved in the increased protein catabolism after burns and during exposure to cold has been discussed.

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THE EFFECTS OF HYPOPHYSECTOMY, GROWTH AND ADRENOCORTICOTROPIC HORMONES ON THE INCORPORATION OF P^{32} INTO LIVER PHOSPHOLIPIDS

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IT HAS been reported that growth hormone causes an increase of liver fat during fasting (Szego and White, 1949; Li, Simpson and Evans, 1949), and that administration of adrenocorticotropic hormone results in a marked increase of fat content in the liver (Baker, Ingle, Li and Evans, 1948; Li, Ingle, Evans, Prestrud and Nezamis, 1949). On the other hand, hypophysectomy does not influence the fat content of the liver (Chaikoff, Entenman, Gillman, and Reichert, 1948). It would, therefore, seem apparent that the anterior lobe of the pituitary must in some way influence fat mobilization and metabolism. The present investigation concerns a study on the phospholipid fraction of the liver and attempts to elucidate the role of anterior pituitary hormones in lipid metabolism.

EXPERIMENTAL

Three separate experiments were carried out. The first was designed to study the effects of ageing on phospholipid P^{32} incorporation: normal male rats (Long-Evans strain, 25, 55, and 240 days of age) were used. The second experiment studied the effect of growth hormone: normal rats were injected with a 2 mg. daily dose of growth hormone for 13 days starting at 40 days of age. The final experiment was conducted in hypophysectomized rats: animals were operated at 40 days of age; injections began on the day of operation with a daily dose of 0.2 mg. of growth or adrenocorticotropic hormone and continued for about 14 days. All animals were fasted 24 hours and received an intraperitoneal injection of 6 microcuries of P^{32}—labelled Na_2HPO_4 solution 2 hours before autopsy. The growth and adrenocorticotropic hormones were prepared by the methods previously described (Li, Evans and Simpson, 1945, 1943).

The animals were anesthetized with nembutal; the livers were extirpated, exsanguinated, and frozen. One gram of liver was homogenized in distilled water at 0°C. to make up a 20% homogenate. Duplicate analyses were carried out with one cc samples. Each sample was subjected to isolation of the acid-soluble and phospholipid phosphorus fractions by modifications of the methods of Schmidt and Thannhauser (1945) and Schneider (1946).

Aliquots of each fraction were taken for phosphorus determinations by the method of Fiske and Subbarow (1925), and for radioactivity measurements using a Tracerlab

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[1] Predoctoral AEC Fellow in Biological Sciences—1948–1949.

"Autoscaler." The activity is expressed in terms of "relative specific activity" (see footnote in Table 1 for definition), with the specific activity of acid-soluble phosphorus in the denominator, as suggested by Zilversmit, Chaikoff and Entenman (1948).

RESULTS AND DISCUSSION

In Table 1, the results obtained in the experiments concerned with the effects of ageing are presented. It is evident that the turnover of liver phospholipids is significantly decreased with ageing. Lang (1937) has stated that the concentration of liver phospholipids increases slightly in rats from

TABLE 1. THE EFFECT OF AGEING ON THE INCORPORATION OF P^{32} INTO PHOSPHOLIPID IN THE LIVERS OF NORMAL ANIMALS

Group	Age	No. of rats	Relative specific activity*	Significance	
				Groups compared	"p"
	Days				
I	25	8	0.211 ± 0.005†	I vs. II	<0.001
II	55	10	0.170 ± 0.007	II vs. III	<0.001
III	240	7	0.113 ± 0.010	III vs. I	<0.001

* Relative Specific Activity = $\dfrac{\text{Phospholipid } P^{32}}{\text{Phospholipid } P^{31}} \Big/ \dfrac{\text{Acid-sol. } P^{32}}{\text{Acid-sol. } P^{31}}$.

† Mean ± standard error.

the day of birth to 45 days of age, while Williams, Galbraith, Kaucher, Moyer, Richards and Macy (1945) have shown a steady increase in the concentration of liver phospholipids from 15 to 70 days of age. Thus the decrease in turnover is apparently accompanied by an increase in the phospholipid concentration in the liver.

TABLE 2. THE EFFECT OF GROWTH HORMONE ON THE INCORPORATION OF P^{32} INTO PHOSPHOLIPID IN THE LIVERS OF NORMAL ANIMALS

Experiment	No. of rats	Relative specific activity	Significance "p"
Control	10	0.170 ± 0.007†	
Injected*	9	0.257 ± 0.014	<0.001

* Male rats hypophysectomized at 40 days of age; injections began on the day of operation and continued for 14 days; a daily dose of 0.2 mg growth hormone was used.

† Mean ± standard error.

Falkenheim (1942) found a decrease in the rate of phosphorus turnover with age in the liver of the growing mouse. Fries, Changus and Chaikoff (1940) working with brain and spinal cord, and Patterson, Keevil, and McHenry (1944), with kidney, have found a decrease with age in the turnover of the tissue phospholipids. Flock, Bollman and Berkson (1948), however, claimed that the turnover rates of liver phospholipids from rats

weighing 75 grams, 123 grams, and 198 grams, were identical. The age of such rats in our colony would be from 32 to 60 days of age, so that neither the extremities, nor the width of our range is covered.

The changes in normal rats as the result of growth hormone administration are given in Table 2. A marked significant increase in the incorporation of P^{32} into phospholipids is observed after growth hormone treatment.

In Table 3, it is demonstrated that neither hypophysectomy, nor hypophysectomy plus growth or adrenocorticotropic hormone changes the concentration of liver phospholipids, yet all three conditions have signifi-

TABLE 3. THE EFFECTS OF GROWTH AND ADRENOCORTICOTROPIC HORMONES ON LIVER PHOSPHOLIPID INCORPORATION OF P^{32} AFTER HYPOPHYSECTOMY

Group	Experiment	No. of rats	Phospholipid P content	Relative specific activity	Significance	
					Groups compared	"p"
			mg. per 100 g. wet liver			
I	Normal	10	126 ± 3	0.170 ± 0.007†		
II	Hypophysectomy	10	125 ± 7	0.087 ± 0.009	II vs. I	<0.001
III*	Hypophysectomy + Growth Hormone	9	126 ± 6	0.116 ± 0.007	III vs. II	<0.02
IV*	Hypophysectomy + ACTH	7	124 ± 6	0.132 ± 0.011	IV vs. II	<0.01

* Male rats hypophysectomized at 40 days of age. Injections began on the day of operation and continued for 14 days; 0.2 mg. daily dose of the hormone was used.
† Mean ± standard error.

cant effects on the phospholipid turnover. Hypophysectomy markedly decreases this turnover, while treatment with growth or adrenocorticotropic hormone causes an elevation. However, under no condition of these treatments is the level returned to normal.

Chaikoff, Gibbs, Holton and Reichert (1936) have reported that in the dog "in the absence of all hypophyseal tissue . . . phospholipid . . . (is) contained in normal amounts in the liver." The results presented in Table 3 confirm this finding.

In P^{32} uptake experiments, Fraenkel-Conrat and Li (1949) found a marked decrease in the relative specific activity of liver phospholipids of hypophysectomized animals, and the administration of growth hormone to such animals effectively elevated the value toward normal. The changes observed by these authors were more marked than ours, which is probably due to their use of the specific activity of plasma inorganic phosphorus to calculate the relative specific activity. These authors also report an increase in the relative specific activity of liver phospholipids after the administration of adrenocortical extract to normal animals. Stillman, Entenman, Anderson, and Chaikoff (1942) have shown, however, that the incorpora-

tion of P^{32} into the liver phospholipid molecule was not affected by adrenalectomy.

SUMMARY

Ageing causes a decrease in the uptake of P^{32} by liver phospholipids.

Hypophysectomy, with or without growth or adrenocorticotropic hormone treatment, does not influence the concentration of phospholipids in liver.

Hypophysectomy results in a decreased turnover of liver phospholipids.

Growth hormone produces an increase in the turnover of liver phospholipids in normal or hypophysectomized rats.

Treatment of the hypophysectomized animal with adrenocorticotropic hormone results in an increased turnover of liver phospholipids.

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EXPERIMENTAL BASIS FOR SELECTING THE OPTIMAL CONDITIONS FOR QUANTITATIVE FLUOROMETRY OF NATURAL ESTROGENS

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WEILAND, Straub, and Dorfmuller (1929) and Marrian (1930) observed that various steroids fluoresced when treated with concentrated sulfuric acid. Later Cuboni (1934) described a simple qualitative method for determining pregnancy in mares based upon this fluorescence. The method consisted of heating benzene extracts of acid hydrolyzed urine with concentrated sulfuric acid, pregnancy being indicated by the green fluorescence in the sulfuric acid phase. This fluorescence is characteristic of natural estrogens. The Cuboni test has been used extensively as a qualitative test for detecting estrogens in the urine of pregnant mares.

Recently Finkelstein, Hestrin and Koch (1947) have described a fluorometric method for the determination of estrogens based on measurement of a green fluorescence developed when estrogens are heated with 90% phosphoric acid instead of sulfuric acid.

The adaptability of the fluorescence obtained upon heating estrogens with concentrated sulfuric acid to quantitative measurement with a fluorimeter has been studied. In developing the assay procedure, the effect of the following experimental variables upon the fluorescence was considered:

1) The effect of the solvent.
2) The effect of sulfuric acid concentration during development of fluorescence and during measurement of fluorescence.
3) The effect of temperature and of duration of heating.

Based upon this study, optimum conditions for fluorescence intensity, stability and reproducibility were combined into a procedure which is as quantitative as the common colorimetric methods for the determination of these steroids (Kober, 1931; Zimmerman, 1935) but which is more sensitive, more rapid and more specific. The method consists of heating the steroid sample with 80% to 90% sulfuric acid and then diluting with less concentrated acid to stabilize the fluorescence. The method has been briefly described by Bates and Cohen (1947). A somewhat similar method, but one which does not develop maximum fluorescence, has been presented by Jailer (1947).

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APPARATUS

All of the data presented herein have been obtained using a Klett Fluorometer, Model 2070. The measurements were made using a Corning 5970 filter as a lamp filter to remove all visible light. A Corning 3389 filter was used as the photocell filter in order to remove all primary light.

Fluorometer tubes, supposedly calibrated, were found to vary as much as 10%. This explains some of the deviations from a smooth curve of the data shown in Figures 2 and 3 which were experiments in which only single samples were used. Duplicate and triplicate samples usually were taken, which, upon averaging, reduced the gross deviations.

Serum pipettes (0.2 ml.) were used for measuring the estrogen samples.

Commercially available solvents and reagents, usually of c.p. grade, were used without further purification.

The concentration of sulfuric acid is expressed throughout the paper on a volume per volume basis, that is, sulfuric acid (90+10) means an acid solution prepared by adding 90 ml. of sulfuric acid, c.p., to 10 ml. of water. The first number always indicates the volume of sulfuric acid and the second number the volume of water.

EXPERIMENTAL RESULTS

The Effect of Solvent

In doing the Kober test it is usually specified that the solvent be evaporated from the test samples before attempting to develop the color. As far as these studies have gone, such evaporation is not, in general, necessary in the development of the fluorescence produced with concentrated sulfuric acid, provided that the volume of the sample used is small; however, when the solvent used is one which reacts with sulfuric acid to develop a colored or fluorescent compound, the solvent must be removed. These statements also apply in general to the Kober type test as modified by Cohen and Bates (1947). An exception in the case of Kober tests is that solvents such as toluene, which produce a haze on dilution of the acid, must be removed before the assay is undertaken.

In the fluorescence method, water, ethyl ether, methanol, ethanol, ethylene dichloride, benzene and toluene have been used successfully, without evaporation, as solvents for the steroid samples. One impure lot of benzene had to be discarded as it developed a large amount of fluorescence when heated with concentrated sulfuric acid under the conditions used for the test. Because acetone and propyl, isopropyl and butyl alcohols or chlorides react with sulfuric acid to produce colored compounds, they can not be used as solvents for test samples unless they are completely removed by evaporation.

Some samples of absolute and 95% ethanol have been found unsuitable for use as solvent. Their suitability can be determined by making two calibration curves with estrone; one with the ethanol present as solvent and one with the ethanol evaporated in a water bath. If the ethanol is satis-

factory, the calibration curves will run through the origin and be superimposable.

It was also found that the addition of small amounts of ethylene dichloride to an ethanol solution or the addition of a little ethanol to an ethylene dichloride solution reduced the intensity of the fluorescence obtained with estrone to less than 50% of that obtained with either of the pure solvents alone or in their absence.

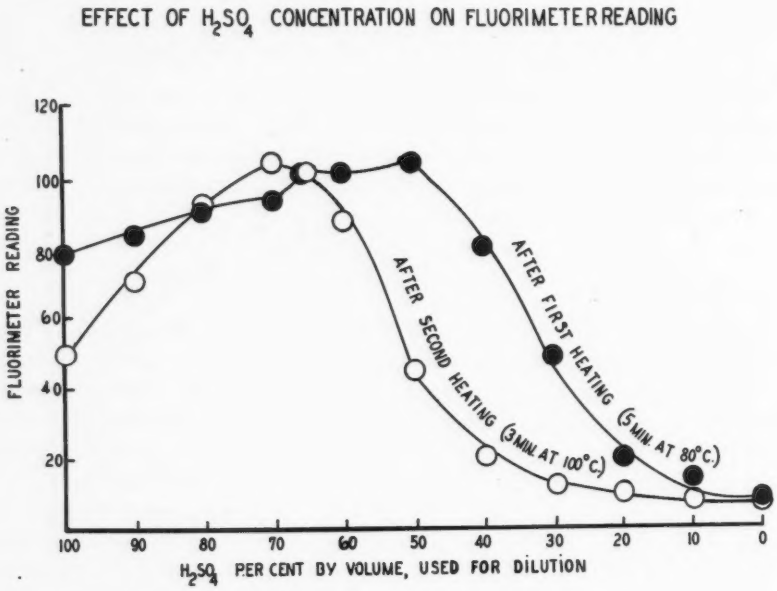

FIG. 1

The Effect of Acid Concentration During the Measurement of Fluorescence

Into a series of fluorometer tubes were pipetted 0.10 ml. samples of an ethanol solution containing 5 micrograms of estrone. To each tube 1 ml. of sulfuric acid (90+10) was added and the tube was shaken. All tubes were heated simultaneously in a water bath for 5 minutes at 80°C. The tubes were removed from the water bath, cooled, diluted with 6 ml. of sulfuric acid of the concentration indicated in Figure 1 and the contents thoroughly stirred. The fluorescence was measured at room temperature and was found to depend on the acid concentration at the time of measurement. The fluorometer readings obtained were plotted in Figure 1 against the concentration of sulfuric acid which was used as diluent.

After these readings were obtained, the same diluted tubes were heated

for 3 minutes in boiling water as in the Kober test. They were then cooled in water to 25°C. and the fluorescence re-determined. The points obtained after this second heating are plotted also in Figure 1.

It is obvious from the data obtained that the maximum fluorometer reading is obtained when 50 to 65% sulfuric acid is used as a diluent. Maximum stability during the second heating is obtained when sulfuric acid (70+30) is used as a diluent. The fluorescence is quenched by dilu-

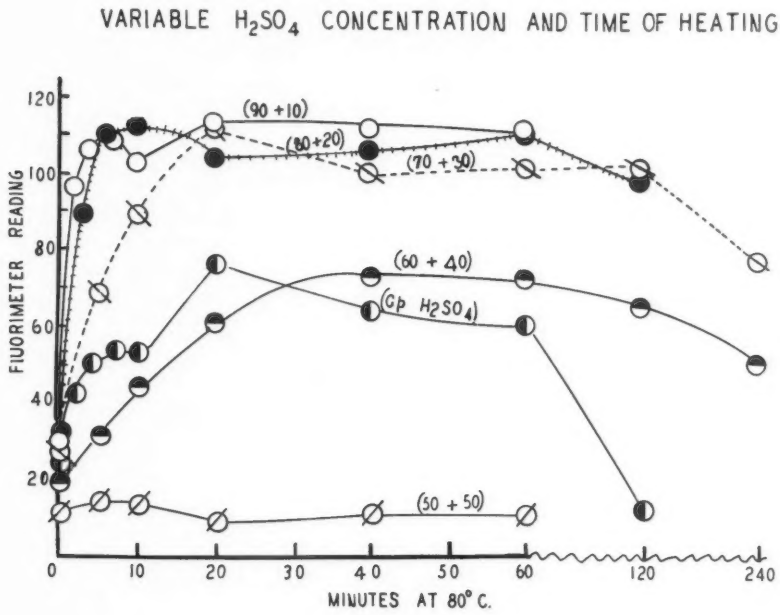

FIG. 2

tion of the acid with water and this quenching is accelerated by heating. On the basis of these findings, we have selected sulfuric acid (65+35) as the diluent.

The second heating has additional significance because it is a step in the Kober test. It is at this stage of the Kober reaction that fluorescence is quenched by dilution with water and parallel with this quenching of fluorescence there is a shift in the color of the solution from yellow to red (1947).

The effect of the sulfuric acid concentration upon the fluorescence at the time of reading is a point which must be kept in mind when interpreting the subsequent data presented. In some experiments fluorescence

was determined in solutions which were not at the optimum sulfuric acid concentration.

The Effect of Acid Concentration During Heating and Duration of Heating

To 0.10 ml. samples of an ethanol solution containing 50 micrograms of estrone were added 10 ml. of sulfuric acid of the concentration indicated in Figure 2. After thorough mixing of the contents, the tubes were heated in a water bath at 80°C. One ml. samples of each mixture (5 micrograms of

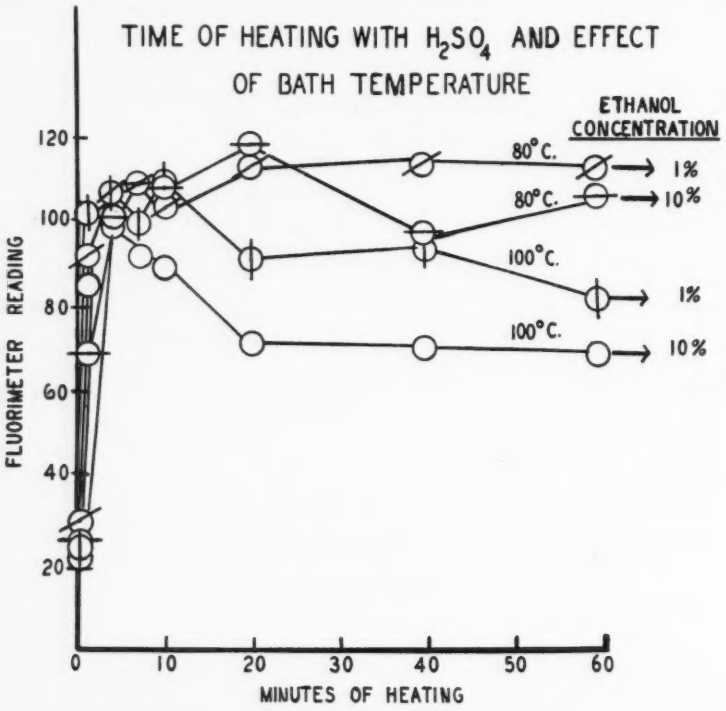

FIG. 3

estrone) were removed before heating and at the various times indicated during heating. Each sample was diluted with 6 ml. of sulfuric acid (65+35), mixed and the fluorescence read. The results are shown in Figure 2.

It is obvious from Figure 2 that sulfuric acid, c.p., either does not develop or more likely destroys part of the fluorescence which is developed. Sulfuric acid (60+40) and (50+50) are not sufficiently concentrated to develop maximum fluorescence, perhaps due to simultaneous

quenching as in Figure 1. Sulfuric acid (90+10), (80+20) and (70+30) all reach the same maximum value for fluorescence. Ten minutes of heating is sufficient to reach the maximum in the case of sulfuric acid (90+10) and (80+20) but 20 minutes are required in the case of sulfuric acid (70+30).

These data indicate that with sulfuric acid (90+10) and (80+20) considerable leeway can exist in the time of heating as long as it exceeds 10 minutes at 80°C.

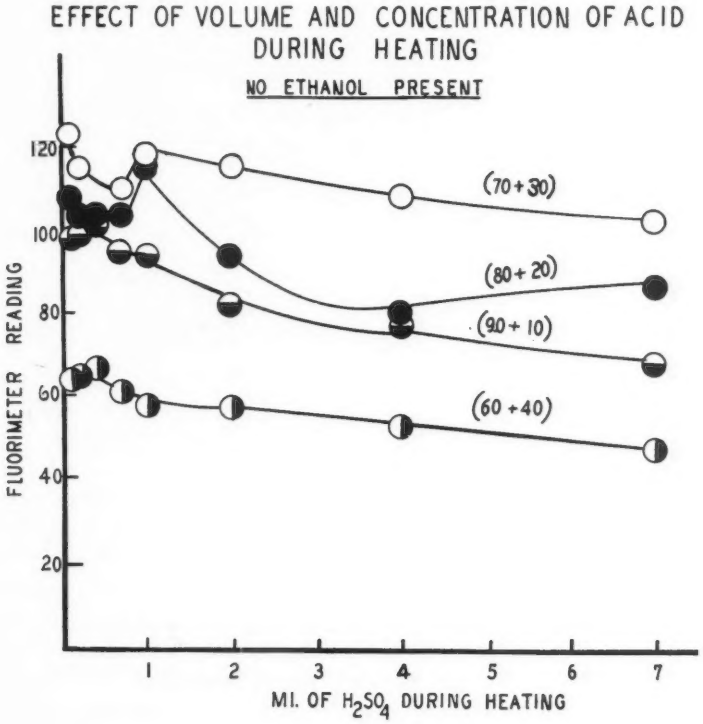

FIG. 4

The Effect of Temperature and Duration of Heating

The data shown in Figure 3 overlap somewhat the data shown in Figure 2 and in subsequent figures. The main point to be shown, however, is the superiority of heating at 80°C. over that of heating at 100°C. Four tubes were made up containing 50 micrograms of estrone in 0.10 ml. of ethanol. To two tubes 10 ml. of sulfuric acid (90+10) were added. To the other two tubes 0.9 ml. of absolute ethanol and 10 ml. of sulfuric acid (90+10)

were added. The data obtained from the tubes with no extra added ethanol were marked 1% on the graph and those containing 0.9 ml. of additional ethanol were marked 10% ethanol. The tubes were placed into a water bath at either 80°C. or 100°C. One ml. samples were removed after the indicated times of heating and were diluted with 6 ml. of sulfuric acid (65+35) for reading. The tubes containing 10% ethanol have correspondingly less fluorescence due to the extra volume entailed.

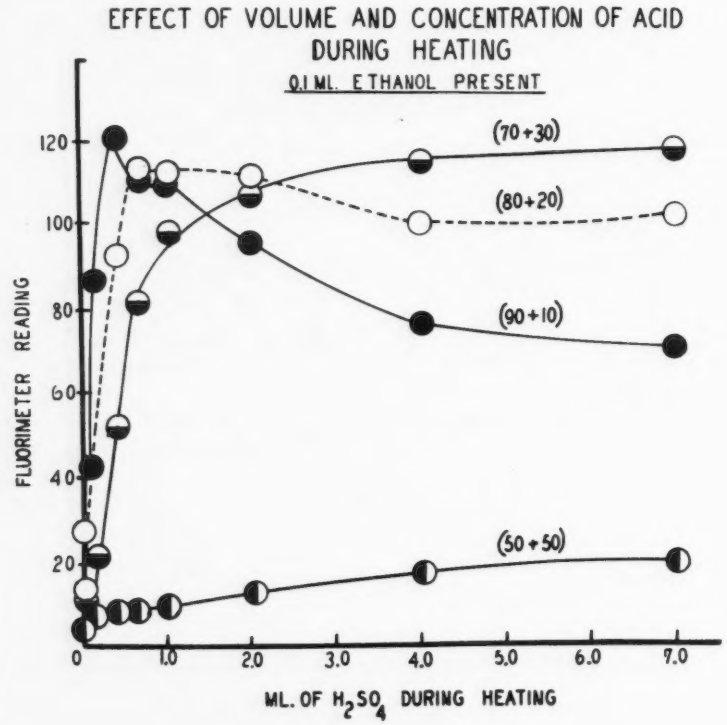

FIG. 5

The results shown in Figure 3 indicate that, after 5 to 10 minutes of heating, all four tubes had approximately the same amount of fluorescence. Upon longer heating at 80°C. the fluorometer readings change little but at 100°C. they fall. The marked stability at 80°C. is the basis for recommending a heating temperature of 80°C. for the development of fluorescence. Slight variation of the temperature, such as a rise of a few degrees above 80°C., will not change the fluorescence much as long as the heating is limited to 10 minutes.

The Effect of Volume and Concentration of Acid During Heating

In all tests of this series, 5 micrograms of estrone in 0.10 ml. of ethanol were pipetted into fluorometer tubes. Either at once (Figure 5) or after evaporation of ethanol (Figure 4), sulfuric acid of the indicated amount and concentration was added, the contents of the tubes thoroughly stirred and the tubes heated 20 minutes at 80°C. After heating, the tubes were diluted with sulfuric acid (65+35) to make a total liquid volume of 7 ml. for reading. Because of the use of sulfuric acid (65+35) for all dilutions, the resulting differences in the final concentration of sulfuric acid at the time of reading caused variation in the magnitude of the fluorescence obtained to the extent indicated in Figure 1.

It is shown in Figure 4 that, in the absence of ethanol, higher readings are obtained with sulfuric acid (70+30) than with other concentrations of sulfuric acid. Also higher readings are obtained in general with smaller volumes of sulfuric acid. The higher values obtained with smaller volumes of sulfuric acid are partly due to the use of sulfuric acid (65+35) for dilution.

Jailer (1947) uses 8 ml. of 60% sulfuric acid and heats for five minutes at 100°C. for the development of fluorescence. The data shown in Figure 4 for sulfuric acid (60+40) indicate that only about 60% of the maximum fluorescence would be obtained by this procedure.

When 0.1 ml. of ethanol is present with otherwise the same experimental set-up (Figure 5) it was found that the smaller amounts of sulfuric acid were diluted so much with the ethanol that maximum fluorescence is not reached. However, maximum fluorescence is reached as soon as 0.5 to 1.0 ml. of sulfuric acid (90+10) or (80+20) is present. Again the drop in the sulfuric acid (90+10) curve is largely due to the difference in fluorescence between the fluorescence in sulfuric acid (65+35), which was the acid concentration at the time of reading with the lower acid volumes, and that in sulfuric acid (90+10) which was the acid concentration at the largest volume.

As previously shown in Figure 2, fluorometer readings of approximately equal values are obtained using either (90+10), (80+20) or (70+30) if 1 ml. of acid and 10 to 20 minutes of heating at 80°C. are used.

The Effect of Amount of Ethanol and Length of Heating

It has been shown in previous sections that maximum fluorescence is obtained when the concentration of sulfuric acid prevailing during the heating period is about (80+20) or (70+30). This concentration can be reached two ways: 1) by the addition of diluted sulfuric acid of proper concentration to a dry sample or 2) by the addition of a more concentrated

acid solution to a wet sample containing the right amount of ethanol or water. It has been our experience that 0.2 ml. of ethanol decreases the sulfuric acid concentration as much as 0.1 ml. of water decreases it.

The data shown in Figures 6 and 7 were obtained in the following manner: 0.1 ml. (5 micrograms) samples of estrone were pipetted into a series of tubes. As indicated, some tubes were heated to remove the ethanol while others had additional ethanol added to them. One ml. of sulfuric acid of the

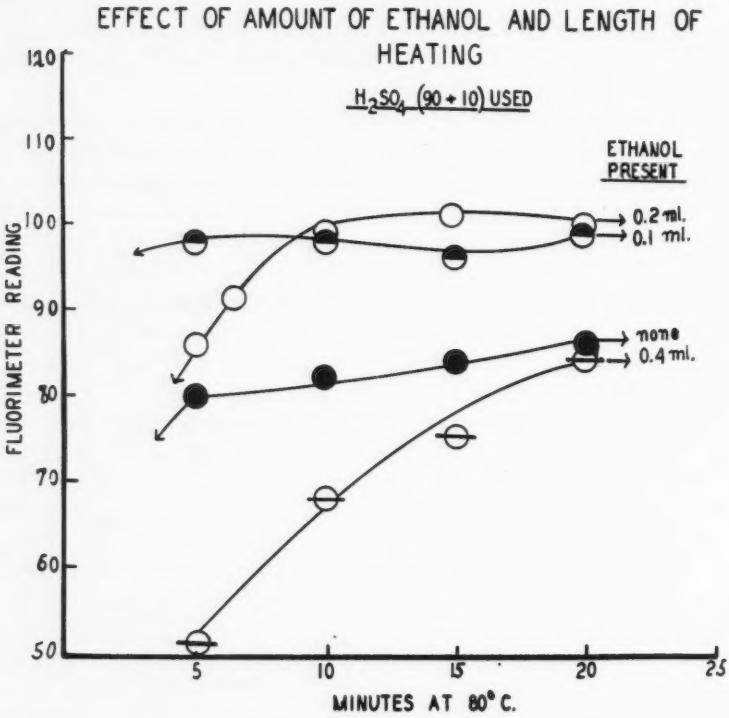

FIG. 6

indicated concentration was added to each tube which was then heated for the time indicated on the graph. After heating, 6 ml. of sulfuric acid (65+35) were added and the solution thoroughly mixed. The fluorescence was then measured as usual.

The data obtained using 1 ml. of sulfuric acid (90+10) during heating with three different quantities of ethanol are shown in Figure 6. Sulfuric acid (90+10) added to the dry sample was found to give lowest values. This is in agreement with the data in Figure 4. A constant reading was

obtained with 0.1 ml. of ethanol present. When 0.2 ml. of ethanol was present with the consequent lower sulfuric acid concentration, the maximum reading was not reached until the sample had been heated for 10 minutes. When 0.4 ml. of ethanol was present the maximum reading was not reached in 20 minutes.

In Figure 7 are shown the results obtained using sulfuric acid (80+20) and (70+30). 0.1 ml. of ethanol dilutes sulfuric acid (70+30) so much that

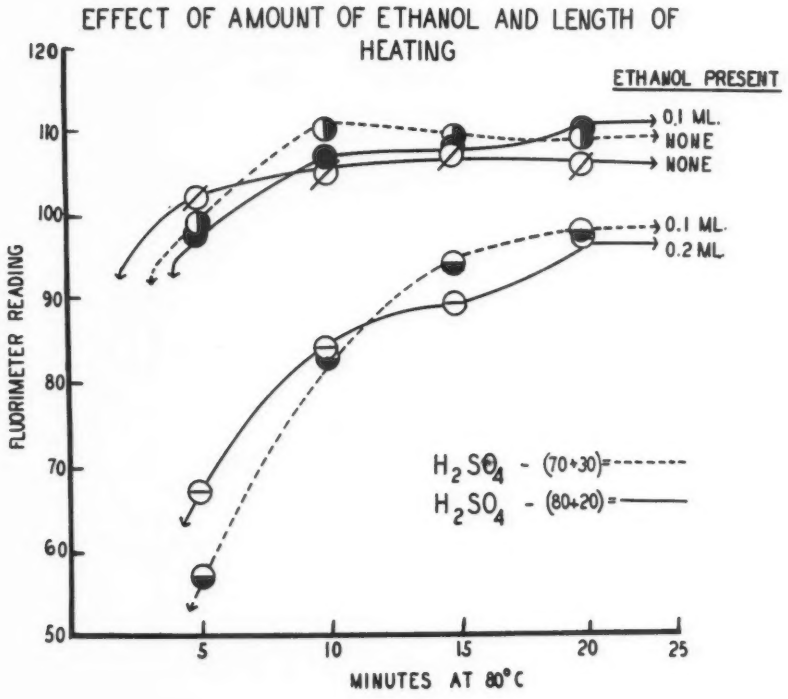

FIG. 7

maximum intensity is not reached even after 20 minutes of heating. 0.1 ml. of ethanol is satisfactory with sulfuric acid (80+20) but 0.2 ml. is too much.

Because of the inconvenience of evaporating samples to dryness and because of our experience in finding greater irregularities and discrepancies between duplicate and triplicate samples after evaporation, we prefer to use sulfuric acid (90+10) and a sample size of 0.1 ml. or 0.2 ml. of ethanol or 0.1 ml. of water. If a sample containing less than 0.1 ml. ethanol is used, additional ethanol is added to bring the solution up to 0.1 or 0.2

ml. volume. Sulfuric acid (80+20) may be used with dry samples. Samples in ethanol or water of a larger volume then those recommended will not give maximum quantitative values but such large samples are rarely necessary because of the sensitivity of the method. Larger samples of benzene or toluene solutions may be used because, unlike alcohol solutions, they contribute no water upon treatment with the sulfuric acid.

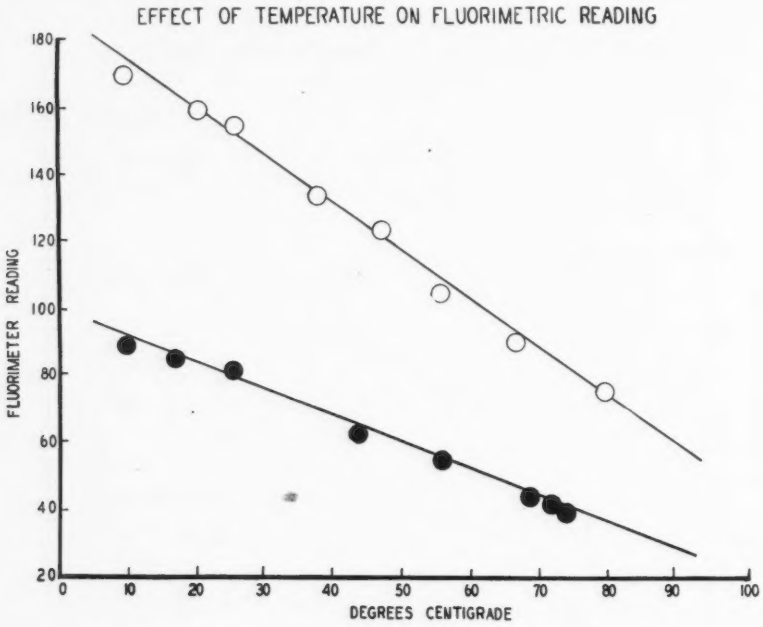

FIG. 8

The Effect of Temperature of the Solution on Fluorometer Reading

Two estrone solutions of different concentrations were heated with sulfuric acid (90+10) to develop maximum fluorescence and then diluted with 6 volumes of sulfuric acid (65+35). Each stock fluorescent solution was then divided among four different fluorometer tubes. The quadruplicate tubes were heated or cooled in a water bath to the various temperatures indicated in Figure 8. The readings given are the averaged values obtained at each temperature. Straight lines have been drawn by inspection through the resulting points and found, by extrapolation, to indicate a zero fluorescence at a temperature of about 125° to 130°C.; this is an observation of unknown significance.

The change in reading with a 1° rise in temperature is apparently directly

proportional to the intensity of fluorescence. With a fluorometer reading around 100 scale divisions at 25°C., the temperature error is about 1% per degree (or 0.05 I.U. of estrone). This is an important source of error and must be kept in mind at all times.

In our work we have made no temperature corrections; we always used the standard solutions of estrone with which we have calibrated the fluorom-

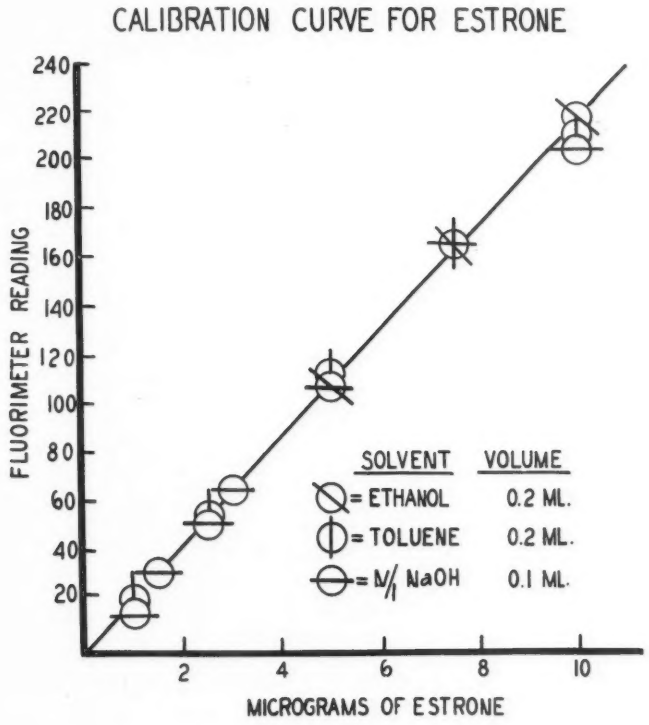

FIG. 9

eter at the same temperature as that of that of the unknowns which were to be measured.

Calibration Curves for Estrone

The calibration curve for estrone, using three different solvents for the estrone, is shown in Figure 9. The total volume of solvent in all tubes within a series was that indicated in the figure. The procedure was to measure the estrone sample carefully into the bottom of the tube, add 1.0 ml. of sulfuric acid (90+10) and heat at 80°C. for 10 minutes. After heating,

6 ml. of sulfuric acid (65+35) were added. The mixture was thoroughly stirred and the fluorescence was determined. Triplicate samples were run throughout and the average values were plotted.

That the fluorescence is quantitatively the same with all three solvents and that a straight line relationship holds, is apparent. The slight decrease of the fluorescence at 10 micrograms of estrone indicates that this is the limiting concentration of estrone which would give a straight relationship. This decrease may be partly due to the color density of the solution as well as to so-called self-quenching.

TABLE 1. RELATIVE FLUORESCENCE OF ESTROGENS, OTHER STEROIDS, AND POLYCYCLIC COMPOUNDS

Compound	Visual color of:		Relative fluorescence
	Transmitted light	Fluorescent light	With 3389 filter
Estrone	Yellow	Bluish Green	100
α-Estradiol	Yellow	Greenish Blue	83
β-Estradiol	Yellow	Bluish Green	94
Estriol	Yellow	Bluish Green	34
β-Dihydroequilenin	Orange	Yellow	60
Equilin	Yellow	Yellow Green	25
Androstenediol 3,17,trans	Colorless	Bluish	5
Dehydroisoandrosterone	Blue	Bluish	4
Progesterone	Colorless	Colorless	<2
Pregnandiol	Colorless	Colorless	<2
Testosterone	Colorless	Colorless	<2
Stilbestrol	Amber	Bluish	3
Hexestrol	Colorless	Colorless	<1
Dienestrol	Colorless	Colorless	<1
Anthracene	Slightly dark	Bluish (unstable)	10
2-Acetyl-9, 10-dihydrophenanthrene	Yellow Green	Green	20
β-Naphthol	Greenish	Green	8
Cholesterol	Yellow Green	Bluish	3
4-Hydroxyphenanthrene	Pink	Pink	3

Fluorescence of Various Compounds

A large number of organic compounds were tested to determine the amount of fluorescence which they will give with sulfuric acid under the conditions described above for estrogens. In all cases 100 microgram samples were used except in the case of estrogenic steroids, when only 5 to 10 microgram samples could be used.

Data on some of the more interesting of the compounds tested are shown in Table 1. It is clearly shown that different natural estrogens have different fluorescence intensities per microgram. When one has a mixture of such estrogens one would get only an average value.

The fluorescence obtained with certain compounds, such as anthracene,

is very unstable in the presence of ultraviolet light, losing 50% of the intensity within a few minutes. A high specificity of this test for the natural estrogens relative to the other steroids tested is obvious. Further specificity among the estrogens can be obtained by using selected filters especially of the interference type (Bates and Cohen, 1950).

Quenching of the Fluorescence

Oxidizing agents such as hydrogen peroxide and nitrates, will destroy the fluorescence once it is developed or will prevent its appearance if they are added first in an adequate concentration. Quantitative dilution studies showed that the addition of 5 micrograms of sodium nitrate or 50 micrograms of hydrogen peroxide to 5 micrograms of estrone in a fluorometer tube will prevent the development of fluorescence.

It is interesting that the quenching curve of a constant amount of sodium nitrate is similar to the stability curve in Figure 1, that is, it takes more nitrate ion to quench the fluorescence in 60% sulfuric acid than in a more concentrated or more dilute acid.

The presence of the sulfite ion is without effect on the fluorescence. Five mg. of salicylic acid was found to prevent the quenching by 5 micrograms of sodium nitrate but was without effect on quenching by hydrogen peroxide. Of many reducing agents tested, only salicylic acid was effective in preventing quenching and it prevented only quenching by nitrates.

Phenol seems to have a specific quenching effect on fluorescence without altering the intensity of the red colored compound formed on heating with sulfuric acid and subsequent dilution with water (Cohen and Bates, 1947).

The Recommended Assay Method

On the basis of the data described in this paper, the following general procedure has been selected. The test sample and a standard preparation are diluted to contain 0.1 to 5.0 micrograms of estrogen per 0.1 or 0.2 ml. using a suitable solvent such as ethanol, toluene or water. Samples of 0.1 ml. of aqueous solutions or 0.2 ml. of ethanol or toluene solutions are accurately pipetted into separate fluorometer tubes. Samples of the same size and in the same solvent are used for both standard and unknown. One ml. of sulfuric acid (90+10) is added to each tube and the tube is shaken. All tubes are placed into a water bath at 80°C. $\pm 5°$ for 10 to 20 minutes. The tubes are removed from the bath and 6 ml. of sulfuric acid (65+35) are added. The contents of each tube are mixed thoroughly using a flattened, spirally-tipped stirring rod. After standing for several minutes to permit the bubbles due to agitation to disappear, the relative fluorescence of the standard and the unknown is determined.

DISCUSSION OF THE PROCEDURE

The following statements can be made on the basis of the above results:

1) The sample must be measured accurately.
2) The final volume must be the same in all tubes.
3) The sulfuric acid concentration during the ten minute heating period, i.e., after dilution by the sample, must be the same in both the standard and the unknown and should be between 70% and 85% sulfuric acid by volume. Because of the breadth of this concentration zone, it is not necessary to cover the tubes during heating.
4) If the sulfuric acid concentration is about 80%, then ten minutes' heating is sufficient and if the acid concentration is about 70% then twenty minutes' heating is required to reach maximum fluorescence values. Because of the stability at 80°C. of the fluorescent compound formed, considerably longer periods of heating, up to one hour, may be used, but are not necessary.
5) The temperature of the bath during heating is not critical. A 5° change during heating will not alter the results.
6) The dilution of the heated sample with sulfuric acid (65+35) brings about an acid concentration at which the fluorescent compound is stable at room temperature for a period of days.

SUMMARY

The experimental basis for a quantitative fluorometric method for the determination of 0.1 to 5.0 micrograms of natural estrogens is given. The method is as follows: Add 1 ml. of 90% sulfuric acid by volume to a 0.1 ml. sample of the estrogen in ethanol, toluene or alkaline aqueous solution in a fluorometer tube. Heat 10 to 20 minutes in a water bath at 80°C. Remove from bath and add 6 ml. of 65% sulfuric acid by volume. Mix thoroughly and determine the fluorescence in parallel with a standard similarly prepared.

With estrone as 100%, alpha-estradiol gives a relative fluorescence of 83%, beta-estradiol of 94%, estriol of 34%, beta-dihydroequilenin of 60% and equilin of only 25%. All non-estrogenic steroids tested gave values of less than 5%.

Acknowledgments

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FLUORESCENCE SPECTRA OF NATURAL ESTROGENS AND THEIR APPLICATION TO BIOLOGICAL EXTRACTS

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INTRODUCTION

IT HAS been demonstrated that microgram amounts of crystalline estrone can be determined quantitatively by fluorometric methods of assay (Bates and Cohen, 1947; Finkelstein, Hestrin and Koch, 1947; Jailer, 1947). The problem of quantitative fluorometric determination of estrogens may be broken down into two parts. 1) The conversion of the steroid into the maximum amount of the compound which is fluorescent is a chemical problem. 2) The measurement of the fluorescence under the best conditions for giving specificity is an instrumental problem. The first, or chemical problem, is discussed by Bates and Cohen (1950). The second, or instrumental problem is the subject of this paper.

There are many fluorescent or potentially fluorescent materials in urine. Some, but unfortunately not all, of these compounds may be fractionated easily from the estrogens. Some of these have natural fluorescence which is not altered appreciably by heating with 90% sulfuric acid. Proper selection of filters can reduce this non-specific fluorescence.

In this paper the best instrumental conditions using a commercial fluorometer and filters for measuring the fluorescence of estrogens are demonstrated. Using interference filters, the fluorometric spectra of various estrogens were measured roughly. Equilin, equilenin and alpha- and beta-dihydroequilenin were found to have markedly different fluorescent spectra from dihydroequilin, estrone, estriol and alpha- and beta-estradiol. Recommendations are presented concerning the best wave lengths for the activation of the fluorescence and for the measurement of the intensity of the fluorescence produced in order to obtain maximum specificity.

APPARATUS

All measurements have been made with a Klettfluorometer, Model 2070, which has two photocells of the barrier-layer type and an H-4 mercury vapor lamp. Within the range of 420 and 600 $\mu\mu$, the photocells have a relative response which varies less than 20% and which is therefore ignored.

Cuvettes (50×50×10 mm.) were used for all measurements of spectra in order to

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increase readings. Fluorometrically calibrated test tubes, 12 mm. in diameter, were used for all other tests.

The following Corning glass filters were used: #5970 with maximum transmission at 365 μμ, #3387 with over 80% transmission at wavelengths above 450 μμ and #3060 with over 89% transmission at wavelengths above 380 μμ.

Numerous interference filters[1] were used. These filters are numbered according to the wavelength at maximum transmission. The filters were found to differ considerably in percent transmission. Because of this variation, the transmission of each filter was measured using a Beckman spectrophotometer and the data were plotted graphically. The areas under the transmission curves were determined for each filter. On the basis of these areas fluorometer readings were corrected to the same relative value for transmission when comparisons were made using different filters, as in the curves of spectra. For example, the 524 μμ filter had a high transmission, so that fluorometer readings obtained were multiplied by a factor of 0.345. The 512 μμ filter, on the other hand, had the lowest transmission and readings obtained with it were multiplied by a factor of 1.3. These were the extreme values of the filters used and are necessary corrections since the one filter transmitted four times as much light as the other.

Interference filters have more than one transmission peak; hence auxiliary filters are necessary under certain conditions. For example, the 540 μμ filter also has a transmission peak at 370 μμ and would transmit some of the primary light reflected from the walls of the test tube if a #5970 filter were used as a lamp filter. Reflected light is much more of a problem with test tubes than with cuvettes.

EXPERIMENTAL RESULTS

The Fluorescence Spectra of Estrogens

Fifty micrograms of each estrogen in a volume of 0.5 ml. of ethanol were heated with 5 ml. of 90% sulfuric acid at 80°C. for 10 minutes to produce the characteristic fluorescent compound. After heating, the solutions were diluted with 30 ml. of 65% sulfuric acid and were thoroughly mixed. The solutions were then transferred to cuvettes for reading. The spectra were measured using a #5970 lamp filter. Eleven different interference filters were placed between the cuvette and the photocell. All readings were made with no change in slit widths and were corrected for blank readings which were obtained using a cuvette filled with 65% sulfuric acid. The fluorometer readings obtained with each interference filter were corrected for the transmission of the filter. The slight dip in the curves at 524 μμ is probably an artefact caused by error in the correction factors used.

Figure 1 shows the great similarity among the fluorescence spectra of the fluorescent compounds obtained from estrone, α-estradiol, β-estradiol and estriol upon heating with sulfuric acid. The last two have only about one-half of the fluorescence intensity of the estrone compound under these conditions. The α-estradiol compound has relatively a greater amount of blue fluorescence (450 μμ) than any of the others, and slightly less fluores-

[1] Obtained from Baird Associates, Cambridge, Mass.

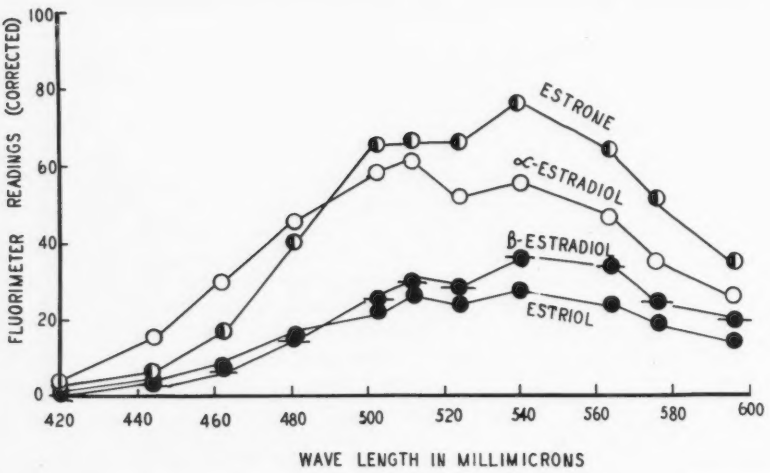

FIG. 1. Fluorescence spectra of compounds formed by heating estrogens with sulfuric acid. A #5970 lamp filter was used in all cases for activation. Eleven different interference filters were used as photocell filters. There were 50 micrograms of each estrogen in a total volume of 35 ml.

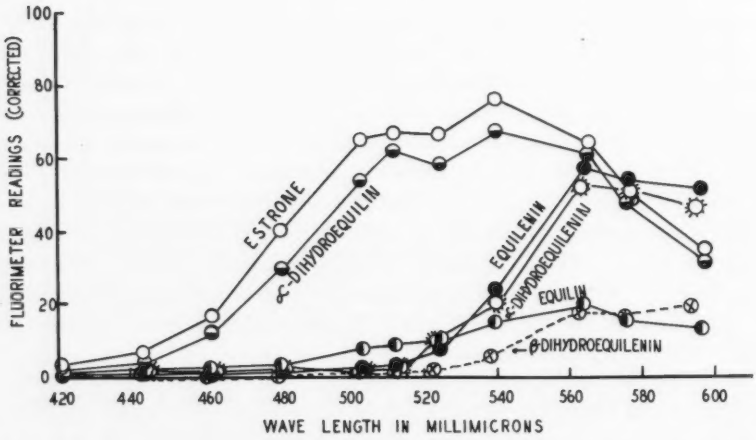

FIG. 2. Fluorescence spectra of compounds formed by heating estrogens with sulfuric acid. A #5970 lamp filter was used in all cases for activation. Eleven different interference filters were used as photocell filters. There were 50 micrograms of each estrogen in a total volume of 35 ml.

cence intensity in the region above 520 $\mu\mu$. This difference is sufficient to be visible to the naked eye as more of a blue color.

Figure 2 shows that equilin, equilenin and the dihydroequilenins differ from the other estrogens in that their fluorescent compounds have negligible fluorescence in the blue region. The compounds obtained from equilin and β-dihydroequilenin have less over-all fluorescence than any of the other estrogens, even in the green and yellow regions; whereas those from equilenin and α-dihydroequilenin have a relatively stronger fluorescence at a wave length around 590 $\mu\mu$. The color of the fluorescence obtained

TABLE 1. THE AVERAGED RELATIVE INTENSITY OF FLUORESCENCE OBTAINED WITH TWO DIFFERENT LAMP FILTERS

There was no change in slits. The fluorometric reading with the 422 $\mu\mu$ lamp filter was divided by the reading with #5970 lamp filter for each of the 10 interference filters used as photocell filters and the ratios averaged. The #5970 L.F. data is that plotted in Figures 1 to 3.

Preparation	$\frac{422 \text{ L.F.}}{5970 \text{ L.F.}}$	Preparation	$\frac{422 \text{ L.F.}}{5970 \text{ L.F.}}$
Quinine	0.13	Weak phenolic fraction[2]	2.7
Weak phenolic fraction[1]	0.15–1.0	Equilin	(3.1–5.0)
Equilenin	2.0	Estrone	3.5
α-dihydroequilenin	2.0	α-dihydroequilin	3.9
α-dihydroequilenin diacetate	2.15	α-estradiol	4.4
β-dihydroequilenin diacetate	2.15	β-estradiol	5.1
		estriol	6.0

[1] Urine from non-pregnant woman.
[2] Urine from woman in late pregnancy.

with equilin and equilenin has a golden appearance; whereas that with estrone is a bluish-green. The spectrum of the fluorescent compound from alpha-dihydroequilin is quite different from that of the equilin compound and very similar to that of the estrone compound.

Effect of Lamp Filter on Spectra of Estrogens

When the fluorescence spectra of the above estrogen solutions were similarly measured, using the 422 $\mu\mu$ filter as the lamp filter instead of the #5970 filter, there was no change in the position or in the shape of the spectral curves. The fluorometer readings, that is, the intensities of the fluorescence, were increased in all cases by a factor which was nearly constant within experimental error for all wave lengths studied and which seemed characteristic for the estrogen. These factors are shown in Table 1. These factors have significance only when compared with each other. They indicate that the activating light intensity is considerably greater with the 422 $\mu\mu$ filter than it is with the #5970 filter.

The only previous reports on the fluorescence spectrum of estrogens are by Bierry and Gouzon (1936) and by Dhere and Laszt (1947). The former

photographed the emission spectra of estrone and estradiol after treatment with sulfuric acid and found a broad band centering at about 575 μμ. The latter authors measured the spectra of various steroids after treatment with neutral methyl sulfate and found results that agree with those in Figures 1 and 2 except that the band for equilin occurred between 531 μμ and 566 μμ. Possible explanations for these disagreements are differences in instrumentation and methods of developing fluorescence.

Fluorescence Spectra of Urine Fractions and Quinine

When the original Kober (1931) colorimetric procedure or any modification is applied to the phenolic fraction of urine extracts, there is found to be present a contaminating substance or substances which are difficult to remove by fractionation and which impart a yellow to brown color to the sulfuric acid solution. Since this colored impurity absorbs light of the same wave length as the estrogens the result is an over-estimation. In the fluorescence procedures this non-specific yellow color does not interfere appreciably because the estrogen concentration is 1/10 to 1/100 of that used for the Kober determinations. But there are present in the phenolic fractions of mares' urine, and more especially from human urine, substances which have a blue fluorescence both before and after or only after heating with sulfuric acid and which do interfere. Stevens and Dick (1946) have described the isolation of a blue fluorescent substance from human urine.

There are two possible instrumental methods of eliminating or correcting for this blue fluorescence. One method is to use activating light of such a wave length that the blue fluorescing material is not activated but the estrogenic compounds are. The other method is to use photocell filters which measure the fluorescence at a wavelength longer than any produced by the blue fluorescent material.

Quinine was selected as a simple example of a blue fluorescent compound and its fluorescence spectrum was measured using the same technique that was used for the estrogens (Figure 3). Quinine does not fluoresce when exposed to visible light nor does it fluoresce appreciably when exposed to the light passing the 422 μμ interference filter. On the other hand, it has a fairly strong fluorescence when the #5970 filter is used.

It is shown in Figure 3 that the fluorescent compounds obtained on treatment of the weak phenolic fraction from normal human urine have spectra similar to quinine. Note that in both the compounds shown in Figure 3, the fluorescence intensity is markedly decreased by changing from a #5970 to a 422 μμ filter. The direction of this change is the opposite of that found with the estrogens. This is also shown in Table 1 by ratios of less than 1. With the human urine fraction, the ratio was only 0.15 in the

blue region but reached a value of 1 at 575 $\mu\mu$. This indicates that the fraction was a mixture of at least two compounds.

There are thus two advantages in the use of the 422 $\mu\mu$ filter as a lamp filter: 1) the sensitivity of the reaction as a test for estrogens is increased because of greater fluorescence intensity (Table 1), 2) specificity is greatly increased because of the failure of this wave length to activate the blue fluorescence of some of the non-estrogenic compounds (Figure 3). Unfor-

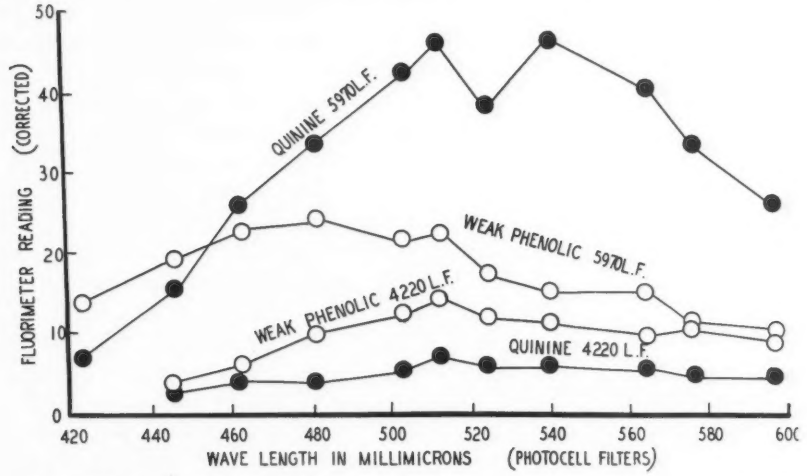

FIG. 3. Showing the effect of the lamp filter on the fluorescence spectra of Quinine and on the weak phenolic fraction from the urine of a non-pregnant woman. A #5970 filter or a 422 $\mu\mu$ interference filter was used as lamp filter as indicated. Eleven different interference filters were used as photocell filters. 100 micrograms of Quinine hydrochloride were dissolved in 35 ml. of 65% sulfuric acid.

tunately, with a mercury vapor lamp such as the H-4, one is limited to those wave lengths at which the mercury produces bands of high intensity. Perhaps if one used a strong light source of still longer wavelength, up to 460–488 $\mu\mu$, an even greater specificity might be obtained.

Another method of correction for the blue fluorescence which is not due to estrogenic compounds would be to measure the fluorescence of the sample added to cold 65% sulfuric acid, i.e., measuring the natural fluorescence of the compound unaltered by heating with sulfuric acid. This blank reading would be subtracted from that obtained after heating. Such a correction procedure necessarily assumes that heating with 90% sulfuric acid does not decrease or increase the fluorescence intensity of the interfering compound. Tests along this line have shown that the fluorescence reading is the same with quinine both before and after heating with 90%

sulfuric acid under the conditions of the fluorometric test. Some tests carried out on phenolic fractions from human urine suggested that such blank corrections might be successfully used but later studies were not so successful. Similar tests with pregnant mares' urine where the blanks were small were also of dubious value.

Use of Auxiliary Filters

Good fluorometric technique requires that a photocell filter or filters be used which completely remove all primary light. With a non-fluorescent solvent in the cuvette or test tube the fluorometer reading will be zero if proper photocell filters are used. In order to obtain this condition when using interference filters as photocell filters, it is necessary to use an auxiliary filter such as a #3387 filter in addition to the interference filter. It has been found repeatedly, especially with test tubes, that high readings are obtained with blank tubes unless such an auxiliary filter is used. This is the result of reflected primary light which is transmitted by many of the interference filters but which is cut out by the #3387 filter. Because the #3387 filter has such a high transmission at wave lengths above 460 $\mu\mu$, its combination does not greatly change the fluorometric reading on the sample and the reading on the blank solutions becomes zero.

When the fluorometer readings were made with the various interference filters, in obtaining the data shown in Figures 1, 2 and 3, readings were also taken with the #3387 filter in combination with the interference filter as photocell filter. The readings thus obtained were, in general, 10 to 20% lower than those obtained without the #3387 filter as would be expected from the transmission of the #3387 filter. They confirmed the readings obtained with the interference filters alone which had been corrected for the blank reading.

Relative Fluorescence Intensities of Various Estrogens Using Test Tubes

Because of the sulfuric acid medium used in these tests, it is more convenient to use test tubes than cuvettes when measuring the fluorescence. Table 2 shows the relative fluorescence of various crystalline estrogens using test tubes and different filter combinations. The values shown are essentially cross sections of spectral curves similar to those in Figures 1 and 2 plus the data with the #3060 photocell filter. Many interesting points are shown in this data. Note that the estriol values are a constant percentage of those of estrone, but that values for all other estrogens change, especially with the lamp filter change. Equilin, equilenin and α- and β-dihydroequilenin give quite low values with B and C filter combinations. Consequently, measurements made with these B or C com-

binations give a fair idea of the estrone, estriol, α- and β-estradiol or α-dihydroequilin content of the solution. On the other hand, values obtained with the filter combinations A or D give a better idea of the total estrogen content of the solution. These comments of course, apply only to the pregnant mares' urine extracts where all the above estrogens might be found.

At the bottom of the table are shown the results obtained when equal amounts of estrone and equilenin were mixed and similarly treated. The

TABLE 2. RELATIVE FLUORESCENCE OF ESTROGENS USING DIFFERENT FILTER COMBINATIONS

5 Microgram Quantities of Each Estrogen Were Treated Identically. Measurements Were Made Using Tubes

Estrogen	Filter combination→A	B	C	D
	Lamp filter→#5970	422$\mu\mu$	422$\mu\mu$	422$\mu\mu$
	Photocell filter→#3060	524$\mu\mu$+#3387	503$\mu\mu$+#3387	596$\mu\mu$+#3387
	Potentiometer Readings With Estrone Set to Read 100			
Estrone	100	100	100	100
α-Estradiol	94	78	78	74
β-Estradiol	95	71	61	101
Estriol	35	35	35	34
Equilin	30	13	7	33
α-Dihydroequilin	97	84	76	102
Equilenin	103	17	12	71
α-Dihydroequilenin	105	6	2	66
β-Dihydroequilenin	55	3	1	31
Estrone 2.5 μg. Equilenin 2.5 μg.	81	53	53	72

values obtained with B and C filter combinations are those which might be expected on the basis of the values obtained on the individual estrogens. For some unknown reason, the values obtained with the A and D filter combinations are significantly lower than would be expected. The reading with the A combinations should be 100 not 81. Such a decrease might be due to some form of quenching or other type of interference but if it were quenching it would be expected to show up with the B and C filters.

Correlation of Fluorometric Results and Bioassay

A criticism often made of the colorimetric or fluorometric assay of estrogens is that each estrogen gives a quantitatively and/or qualitatively different response and only bioassay is reliable. The facts, however, indicate that bioassays of natural extracts also lack as much, if not more, in specificity and quantitative value. α-estradiol is often claimed to be ten times as potent as estrone and estrone is about six times as potent as estriol. This

makes α-estradiol 60 times as potent as estriol according to the usual assay procedures. By making fluorescent readings at different wavelengths as in Table 2, a fairly good estimate can be made of the type of estrogens which are present. The maximum differences in quantitative response among the estrogens is only about three-fold with filter combination A and D. Of course, the combination of bioassay with various chemical methods of assay, such as the fluorometric, Kober and Zimmermann methods, will give more information concerning the estrogens in an extract than any one method could give.

TABLE 3. CORRELATION OF FLUOROMETRIC POTENCY WITH BIOASSAY USING TWO FILTER COMBINATIONS

Fraction		Fluorometric potency		Bioassay‡ potency
		A filter* combination	B filter* combination	
		μg./ml.†	μg./ml.†	μg./ml.†
From Weak Phenolic Fraction From the Urine of Pregnant Mares				
616	Ketonic	36,300	29,580	33,435
	Non-ketonic	25,900	14,000	27,130
8747	Ketonic	16,000	12,500	15,870
	Non-ketonic	22,500	12,300	54,340
M	Ketonic	1,575	1,087	1,145
	Non-ketonic	1,363	138	975
Weak Phenolic Fraction From Urine of Non-pregnant Woman				
W		78	6.3	10.8

* A. Filter = #5970 lamp filter and #3060 photocell filter.
B. Filter = 422μμ lamp filter and 524μμ plus #3387 photocell filters.

† Potency expressed as micrograms equivalent of estrone, since estrone was used as standard.

‡ Modified procedure of Kahnt and Doisy (1928).

In Table 3 is shown the fluorometric results on some typical urine fractions. When the weak phenolic fraction of pregnant mares' urine is separated into a ketonic and non-ketonic fraction, it is found that about half of the fluorometric potency is in each fraction as determined by the A filter combination. The same is not always true with the B filter determinations. Bioassays show that about one-half or even more of the potency is in the non-ketonic fraction. The ketonic fraction, which is crystalline in nature and which contains mostly estrone, gives potency values with both the A filter and the B filter which agree fairly closely with the bioassay potency. In contrast to this the bioassay values of the non-ketonic fraction are two or more times higher than the B filter values which measure only the content of estradiols and dihydroequilins. This greater activity by bioassay is not surprising since α-estradiol and α-dihydroequilin are many times more potent biologically than estrone. With the non-ketonic fraction in all three cases, the A filter gives a much higher value than does the B filter. This is indicative of the high relative content of di-

hydroequilenin which may be estimated to be present to the extent of 50% by weight in the cases of fractions 616 and 8747 and to a still greater extent in the case of fraction M. The dihydroequilenins are physiologically less potent than estrone.

That human urine extracts are considerably different from mares' urine extracts, is shown in the test of the weak phenolic fraction from a non-pregnant woman. Here the A filter value is a much higher value than is the B filter or the bioassay value. The high A reading is due to the blue fluorescent material in the urine. The value obtained with the B filter is near the true value as indicated by bioassay potency. Since human urine contains no equilin or equilenin compounds the interpretation of the data is much simplified. Separation of the estriol from the estrone-estradiol fraction makes it possible to use estriol as the standard for the strong phenolic or estriol fraction and estrone for the weak phenolic fractions. Thus fairly accurate measurements of estrogen content by fluorometric procedure are easily obtained on extracts from human urine.

Recommendations on Use of Filter Combinations

One difficulty with the use of interference filters is the sacrifice of sensitivity which one makes because of the low transmission of the filters. For maximum sensitivity (not specificity) one can use as photocell filters the Corning glass filters such as the #3060 filter with a #5970 lamp filter or a #3387 filter with a 422 $\mu\mu$ lamp filter. The latter filter combination is essentially that used by Jailer (1947, 1948) and Finkelstein (1948). When one uses such photocell filters, which have a high transmission (over 80%) one measures the summation of all the fluorescence whether due to estrogens or impurities, which is longer than a certain wavelength. Because these filters permit the passage of so much of the fluorescent light to the photocell, fluorometer readings of the order of 10 times those obtained with an interference filter are found. Consequently, fluorometric determinations on 0.1 to 0.5 micrograms of estrone may be obtained. When interference filters are used the amount of estrogens required is ten times larger, or 1 to 5 micrograms.

For maximum specificity it is recommended that an interference filter or a glass filter (such as a Corning #5113) transmitting in the region of 420 $\mu\mu$ and having a high transmission, be used as lamp filter. As a photocell filter, an interference filter transmitting in the region of 500–520 $\mu\mu$ with an auxiliary filter, would measure the fluorescence of the compounds obtained from estrone, estriol, α- and β-estradiol, α-dihydroequilin and probably β-dihydroequilin.

By also measuring the fluorescence using as photocell filter a #3387 filter or an interference filter transmitting in the region of 580–600 $\mu\mu$ with an

auxiliary filter, and then by taking the difference between these fluorescence measurements and those with a 500 $\mu\mu$ filter an estimate of the content of equilin, equilenin and α- and β-dihydroequilenin can be made.

SUMMARY

The fluorescence spectra of nine different natural estrogens after heating with sulfuric acid, were measured using interference filters as photocell filters. The spectral curves were of two types. The spectra of estrone, α- and β-estradiol, estriol and α-dihydroequilin were similar. The spectra of equilenin, α- and β-dihydroequilenin and equilin were also similar but markedly different from those of the estrone group.

The fluorescence intensity with compounds of the equilenin group of estrogens was nearly zero with a 500 $\mu\mu$ interference filter as photocell filter whereas the intensity with compounds of the estrone group of estrogens was maximal for each compound. The fluorescence intensity of the equilenin group was maximal in the region of 560–600$\mu\mu$.

With urine extracts it is recommended that a lamp filter transmitting at 420 $\mu\mu$ be used to avoid activation of non-estrogenic compounds that have a blue fluorescence when irradiated with ultraviolet light.

For a photocell filter a glass filter of high transmission over a wide range, such as a Corning #3387 filter, permits determinations of estrogens in the amounts of 0.1 to 0.5 microgram. For greater specificity, interference filters of various wavelengths must be used as photocell filters and 1 to 5 microgram amounts of estrogen are required.

Acknowledgments

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THE LACK OF EFFECT OF THE ADRENAL HORMONES UPON GASTRIC ACID SECRETION[1]

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TUERKISHER and Wertheimer (1945) found that the secretion of free and total acid by the stomachs of adrenalectomized, anesthetized and laparotomized rats was lower than that in control rats. Acid secretion in their preparations was not increased by administration of sodium chloride or desoxycorticosterone acetate, but it was increased by injections of a whole extract of the adrenal cortex. They concluded that the presence of hormones of the adrenal cortex is essential for the secretion of acid by the stomach.

Many of the numerous physiological changes which occur in animals as the result of adrenalectomy are known to affect the ability of the stomach to secrete acid. Among these changes are a fall in blood pressure and cardiac output, a decrease in blood glucose concentration, a distortion of the plasma electrolyte pattern and the occurrence of acidosis. It is possible that the failure of the stomach to secrete acid in the experiments of Tuerkisher and Wertheimer was caused by these abnormalities together with the further insults of anesthesia and laparotomy and not by the absence of adrenal hormones necessary for the secretory process. We have developed methods by which acid secretion can be studied in vitro under standard conditions (Davenport and Chavré, 1950). In using these methods the ability of the parietal cells of the stomach to secrete acid is studied independently of most of the secondary effects of experimental procedures. Accordingly, we have used them to determine whether the function of the parietal cells depends upon or is affected by the hormones of the adrenal cortex.

METHODS

A complete description of the methods has been published elsewhere (Davenport and Chavré, 1950), and it has been shown that the conditions used result in perfect preservation of the cytological structure of the stomach and that they promote maximum secretion of acid.

Young mice of the C57 Black strain obtained from Carworth Farms were used. The esophagus and stomach were removed from a decapitated mouse, and the esophagus

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was tied over a blunt hypodermic needle attached to a syringe filled with phosphate-buffered salt solution. The stomach was washed out with the solution, and the pyloric sphincter was ligated. The stomach was inflated with the solution to a pressure of 75 cm. H_2O, and the cardiac sphincter was ligated. The inflated stomach was placed in 4 ml. of the same salt solution contained in a weighing bottle, and it was incubated for 1 hr. at 38°C. under a partial pressure of oxygen of 3200 mm. Hg. At the end of the period of incubation the fluid was removed from inside the stomach, and its volume was measured. It was placed in contact with a glass electrode system and titrated with standard NaOH solution to the original pH of the buffer. It was then analysed for lactic acid by the method of Barker and Summerson (1941). The difference between the total acid found by titration and the lactic acid was taken to be the inorganic acid secreted.

The salt solution always contained 0.02 M. glucose and, unless otherwise stated, 0.1 mg.% carbaminoylcholine.

At the end of the experiment the forestomach and the stumps of the esophagus and duodenum were trimmed from the secreting part of the stomach whose dry weight was obtained. There is no correlation between dry weight and acid secretion when the dry weights fall between 15 and 25 mg. as they did in these observations, and no attempt was made to reduce the results to the basis of a standard dry weight. Instead, the results are reported as micromoles of acid secreted per stomach per hour.

The rate of acid secretion is affected by the nutritional status of the mice used (Davenport and Jones, 1949). The adrenalectomized mice used in these experiments did not eat normally during the first 48 hrs. after operation. Consequently, stomachs from mice fasted to the same extent as the adrenalectomized mice were used as controls. The groups of stomachs from the adrenalectomized mice and from their fasted controls had the same mean dry weights.

All experiments were performed on female mice, and most of them were repeated on male mice. There was no difference in the responses of the stomachs from the two sexes to the experimental procedures, and the data were pooled:

RESULTS

Mice were adrenalectomized under ether anesthesia, and the rate of acid secretion of their stomachs was measured at intervals thereafter. The results are given in Table 1. In this and the subsequent table the means are

TABLE 1. RATES OF ACID SECRETION IN MICROMOLES OF ACID PER STOMACH PER HOUR BY STOMACHS FROM NORMAL, SHAM OPERATED, ADRENALECTOMIZED OR FASTED MICE INCUBATED IN VITRO

Normal mice	22.0 ± 0.2 (157)
Sham operated 4 hrs.	22.6 ± 0.6 (10)
Adrenalectomized 4 hrs.	21.2 ± 0.7 (14)
Sham operated 24 hrs.	21.9 ± 1.1 (10)
Normal mice fasted 24 hrs.	18.4 ± 0.4 (12)
Adrenalectomized 24 hrs.	18.6 ± 0.3 (21)
Sham operated 48 hrs.	22.3 ± 0.9 (10)
Adrenalectomized 48 hrs., eating	21.6 ± 1.1 (8)
Normal mice fasted 48 hrs.	15.5 ± 0.6 (10)
Adrenalectomized 48 hrs., fasting	16.7 ± 0.5 (16)
Adrenalectomized 7 days	20.8 ± 1.0 (10)

accompanied by the standard error of the mean, and the figure in parentheses is the number of observations.

The secretion by stomachs from mice adrenalectomized 4 hrs. before the observations can be compared with that by stomachs from normal or sham operated mice. The secretion by stomachs from mice adrenalectomized 24 hrs. cannot be compared with the secretion by stomachs from normal or sham operated mice, for most of the adrenalectomized mice did not eat in the first 24 hrs. after operation. Accordingly, a control group of observations on stomachs from mice fasted 24 hrs. is provided. The mice adrenalectomized 48 hrs. fell into two groups, those which ate well during the second day after operation and those which ate little. The former group can be compared with normal control mice or with 48 hr. sham operated mice. A control group of mice fasted 48 hrs. is provided for the latter group. Stomachs from mice adrenalectomized 7 days were also used, and their rate of secretion can be compared with that of stomachs from normal mice.

The data in the table show that there is no difference between the rate of acid secretion by stomachs from adrenalectomized mice and by stomachs from the appropriate controls. Acid can be secreted by the parietal cells in the absence of the hormones of the adrenal glands, and the rate of acid secretion in the absence of the hormones is the same as in their presence.

Desoxycorticosterone has numerous effects upon inorganic ion metabolism, and in order to determine whether it has any effect upon acid secretion desoxycorticosterone as the free alcohol was added to the solution with which the stomachs were filled. When the compound was at a concentration of 5 mg. % the rate of acid secretion was 14.5 ± 0.7 μM per stomach per hr. This is significantly lower than the control rate of 22.0 ± 0.2 μM per stomach per hr. The compound in concentrations ranging from 0.0001 to 1 mg. % had no effect upon the rate of acid secretion in 98 observations. Similar experiments were performed in which cortisone in the form of the free alcohol was added to the fluid in concentrations ranging from 0.0001 to 1 mg. %. In 74 observations this compound had no effect upon the rate of acid secretion.

Because the effects of desoxycorticosterone and cortisone might depend upon the substances used to stimulate acid secretion the observations reported in Table 2 were made. Desoxycorticosterone and cortisone, both as the free alcohols, were added to the fluid with which the stomachs were filled. Stimulating drugs were omitted in one set of experiments, and in the other sets carbaminoylcholine or histamine in optimal concentration was used. The observations were made on stomachs from normal mice and from mice adrenalectomized 24 hrs.

TABLE 2. RATES OF ACID SECRETION IN MICROMOLES PER STOMACH PER HOUR BY STOMACHS FROM NORMAL AND ADRENALECTOMIZED MICE INCUBATED IN THE PRESENCE OF CARBAMINOYLCHOLINE, HISTAMINE, DESOXYCORTICOSTERONE AND CORTISONE

Compound added	No stimulant	0.1 mg. % carbaminolylcholine	10 mg. % histamine
	Stomachs from normal mice		
None	9.3±0.4 (29)	22.0±0.2 (157)	22.7±0.8 (15)
0.1 mg. % desoxycorticosterone	11.2±0.5 (10)	22.4±1.2 (20)	19.5±0.6 (10)
1 mg. % cortisone	8.8±0.5 (5)	20.5±0.6 (15)	21.9±0.8 (5)
	Stomachs from mice adrenalectomized 24 hrs.		
None	9.3±0.7 (9)	18.6±0.3 (21)	17.2±0.5 (9)
0.1 mg. % desoxycorticosterone	8.6±1.2 (5)	16.2+0.5 (10)	16.0±0.7 (5)
1 mg. % cortisone	7.8±0.5 (10)	17.4±0.7 (5)	18.4±1.4 (5)

The data show that in the absence of a stimulating drug desoxycorticosterone and cortisone have no significant effect upon acid secretion. They likewise have no effect when histamine is used to stimulate acid secretion. The data also show that the compounds do not increase the rate of acid secretion of stomachs from adrenalectomzied mice. This is further evidence that the small reduction of the rate of secretion in these stomachs is not to be attributed to the absence of the adrenal hormones.

These observations show that the absence of the adrenal hormones has no effect upon acid secretion, and they also show that desoxycorticosterone and cortisone have no effect in vitro. However, it is possible that the adrenal hormones in large quantities have some effect in vivo which promotes or inhibits acid secretion. In order to test this, normal mice were injected subcutaneously with desoxycorticosterone acetate 14 to 16 hrs. and again with an equal dose 2 hrs. before the stomachs were removed for measurement of acid secretion. The total doses of the compound used were 2, 4 and 8 mg. per mouse weighing between 15 and 20 gms. In 30 observations the rate of acid secretion was found to be identical with that of control stomachs. Similar observations were made using a total dose of 2 mg. of cortisone acetate per mouse. No effect was found in 29 observations.

DISCUSSION

In these experiments the ability of the mouse stomach to secrete acid has been studied in vitro under standard conditions. By this means the acid secreting mechanism has been freed of all effects of adrenalectomy except the absence of the adrenal hormones and the results of poor appetite immediately after the operation. The effects of poor appetite have been controlled by fasting normal mice. Consequently, the effects of the absence of the adrenal hormones should be revealed. Because the rate of acid secretion by stomachs from adrenalectomized mice is the same as that

by stomachs from control mice, we conclude that the adrenal hormones are not essential for the normal function of the parietal cells. With the exception of the inhibitory effect of desoxycorticosterone in the very high concentration of 5 mg. % no inhibitory or stimulating effect of desoxycorticosterone or cortisone upon acid secretion of stomachs from normal or adrenalectomized mice could be found. Since these two compounds appear to have qualitatively all the properties of the hormones of the adrenal cortex, we conclude that these hormones have no role in the acid secreting mechanism. We attribute the reported reduction of acid secretion in adrenalectomized animals to the manifold effects of adrenalectomy upon other physiological processes which in turn affect the secretory ability of the stomach.

SUMMARY

The rate of acid secretion by mouse stomachs has been studied in vitro. The rate of secretion by stomachs from adrenalectomized mice was found to be identical with that by appropriate control stomachs. Desoxycorticosterone and cortisone added to the incubation fluid in a wide range of concentrations had no effect upon acid secretion with the exception that desoxycorticosterone at 5 mg. % concentration inhibited secretion. Desoxycorticosterone and cortisone when injected into normal mice had no effect upon secretion of acid. It is concluded that the hormones of the adrenal cortex play no direct or important part in the mechanism by which acid is secreted.

Acknowledgment

Desoxycorticosterone was generously supplied by the Ciba Pharmaceutical Company through the courtesy of Dr. Ernst Oppenheimer. We wish to thank Dr. Thomas F. Dougherty for his help and Dr. Hans Reich for hydrolyzing cortisone acetate.

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2 P.M. (T) 5 A.M.

A 24-HOUR PERIODICITY IN THE "LH-RELEASE APPARATUS" OF FEMALE RATS, DISCLOSED BY BARBITURATE SEDATION[1]

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INTRODUCTION

UNTIL very recently the importance of the nervous system in evoking the ovulatory discharge of gonadotrophin has been recognized in only the few species of animals which fail to ovulate spontaneously. Within recent months, however, several reports from this laboratory (Sawyer, Everett and Markee, 1948, 1949; Everett, Sawyer and Markee, 1948, 1949; Everett and Sawyer, 1949, a, b; Sawyer, Markee and Everett, 1949, 1950) have demonstrated that in the rat, a spontaneous ovulator, neurogenic activation of the hypophysis is essential for the release of ovulating hormone. It was found that either Dibenamine or atropine, agents which block reflexogenic stimulation of the hypophysis in rabbits (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949), will likewise prevent LH release in rats when injected before certain critical hours during proestrus. In our 4-day cyclic rats these hours are surprisingly uniform, usually between 2 and 4 P.M. Thus, atropine given at 2 P.M. or earlier uniformly prevents LH discharge. When it is given at 4 P.M., however, no interference usually results, ovulation occurring as normally expected during the following night (Everett, Sawyer and Markee, 1949). Similar results have been found with Dibenamine.

It is apparent, therefore, that somewhat as in rabbits, LH discharge in rats depends on neurohumoral stimulation of the adenohypophysis, presumably by an adrenergic agent operating over the hypophyseal portal circulation (cf. Harris' review, 1948; Everett, 1950; Sawyer, Markee and Everett, 1950). Not improbably such mechanisms also operate in other "spontaneously" ovulating species. The property of spontaneity apparently does not reside in the hypophysis⇌ovary system itself as many have supposed, but probably in the hypothalamus. The likelihood of nervous intervention is emphasized by the close relationship between activation of the rat hypophysis and time of day.

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Evidence of a diurnal rhythm of sensitivity in the neural LH-release apparatus of rats was recently described (Everett, 1949, a; Everett and Sawyer, 1949, b) in a study of the induction of LH release by progesterone in 5-day cyclic rats. In such animals there is a transient period of sensitivity to progesterone during the late afternoon of the third day of diestrus, approximately 24 hours before the normal time of neurogenic activation of the hypophysis.

A quite different procedure described herewith provides complementary evidence of this 24-hour rhythm. The results demonstrate the following principal facts: (1) In 4-day cyclic rats, the administration of a moderate anesthetic dose of Nembutal at 2 P.M. during proestrus consistently blocks LH release for a full day, while the same dosage fails to block when injection is delayed until 4 P.M. (2) More prolonged Nembutal sedation on the succeeding 2 days, beginning at the *same critical hour* each day, will usually result in persistence of the graafian follicles with continued estrogen secretion. (3) If on any of these 3 days, treatment is omitted or postponed until after 4 P.M., ovulation will result during the following night. (4) Other barbiturates can reproduce these results, the longer acting ones being effective at dose levels which produce a barely perceptible ataxia. (5) With continued daily treatment the initial set of follicles becomes atretic after 2 to 3 days and the vaginal smears usually return briefly to the diestrous condition.

The results with Nembutal have been partially summarized in a preliminary report (Everett and Sawyer, 1949, a).

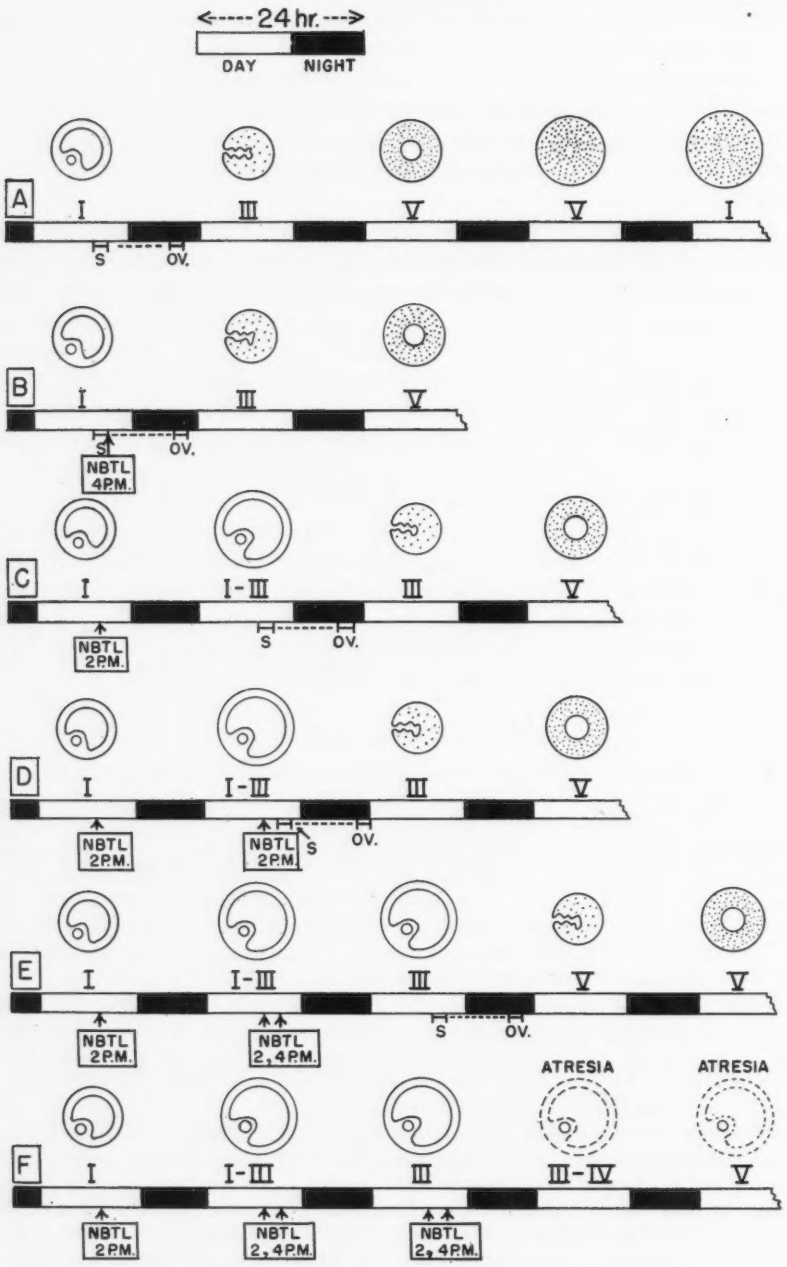
MATERIALS AND METHODS

The animals were adult females of the Vanderbilt (Osborne-Mendel) strain, substrain Va (cf. Everett, 1948; Everett, Sawyer and Markee, 1949). Altogether, 142 rats are represented in the various experimental series, 93 being used in the definitive Nembutal experiments. All experiments were begun on the day of proestrus during presumptive 4-day cycles. None of the animals had previously been treated with barbiturates. A history of at least two 4-day cycles in sequence immediately preceding the experimental cycle was required of each rat. We have previously stated (loc. cit.) that the regularity of cycles in this stock is such that under these circumstances there is a probability of 93% that the next cycle will also be of this length.

Ovulation in these animals occurs with regularity between 1 and 2:30 A.M. (Everett, 1948), 9 to 11 hours after hypophyseal activation on the day of proestrus (Everett, Sawyer and Markee, 1949). Hence, autopsy at convenient hours (8 A.M or later) on the morning after presumptive ovulation, allows an accurate estimate of the efficiency of blocking agents administered on the previous day.

Colony lighting is controlled by a time switch, the lights being on for 14 hours each day. "Midnight" on the colony time scale is the midpoint of the dark period and all reported hours are reckoned from that.

Immediately before autopsy each animal was killed with illuminating gas. The ampullae of the oviducts were excised, compressed in physiological saline under a cover slip and examined under a compound microscope for tubal ova (Everett, 1947). The con-


<-----24 hr.--->
DAY
NIGHT
A
I
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NBTL 4P.M.
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NBTL 2,4P.M.

dition of the uteri was noted, particularly as to whether they were in the distended proestrous condition. The ovaries were removed to physiological saline for observation under the dissecting microscope. The presence or apparent absence of unruptured follicles was observed before fixation in Zenker's fluid. Routinely both ovaries were serially sectioned unless ovulation had obviously taken place and there was no need of assessing the age of all the new corpora lutea. Then only one ovary was sectioned. The routine staining procedure was a modified Mallory tri-acid technique (Everett, 1943).

In the definitive experiments pentobarbital sodium (Nembutal, Veterinary grade, Abbott) was the principal blocking agent employed. This was administered intraperitoneally as an aqueous solution containing 60 mg./ml. in 20% propylene glycol and 10% alcohol. Phenobarbital, barbital, dial and amytal were administered as the sodium salts in physiological saline, at a concentration of 40 mg./ml., prepared on the day of use. For special purposes in one series, atropine sulphate was required as a blocking agent. This was dissolved in Ringer-Locke solution at a concentration of 70 mg./ml. The standard subcutaneous dose was 700 mg./kg. which is known to be effective in blockade of the LH-release apparatus for at least 24 hours (Everett, Sawyer and Markee, 1949). It is judged to become effective within 10 minutes after injection (Everett and Sawyer, 1949. b).

RESULTS

The major Nembutal experiments and their characteristic results are graphically summarized in figure 1, B–F. Attention is called to the final sequence (F) in which it is seen that injection of Nembutal at 2 P.M. during proestrus and at 2 and 4 P.M. on each of the next 2 days results in persistence of graafian follicles for 2 to 3 days beyond the normal ovulation time. Continued estrogen secretion is evidenced by prolonged vaginal cornification. Later the follicles become atretic and vaginal diestrus returns. The procedure shown here was arrived at through a progressive day-by-day analysis, the important details of which are given below.

As shown in table 1, 2 dose levels of Nembutal were employed: 50 mg. and 30 mg. per kg. body wt., respectively. After the exploratory experiments the lower dose became standard. The 50 mg./kg. dose caused profound anesthesia which was maintained for at least 2 hours. The 30 mg./kg. dose characteristically induced what may be termed moderate anesthesia. After injection of this amount, ataxia was always pronounced within 5 minutes and the animals were quiet shortly thereafter. Although spontaneous movement was often absent for the remainder of the first hour, the

FIG. 1. Schematic representations of the normal 4-day cycle (A) and of the characteristic results of different regimes of Nembutal treatment (B–F). Stages of the vaginal smears are indicated by the Roman numerals above each time scale, while the symbols above these show the corresponding follicle and corpus luteum stages. The device marked "S" defines the time limits of pituitary activation as experimentally determined. The device marked "OV" indicates the normal ovulation time in A and the estimated ovulation time elsewhere, arbitrarily assuming a constant interval after pituitary activation. "NBTL"—intraperitoneal injection of Nembutal (30 mg./kg.).

corneal reflex was usually present and movements of the limbs and trunk could be elicited by stretching the forelimbs. Signs of recovery appeared about 1 hour after injection. Although still somnolent the animal would occasionally drag herself to another part of the cage. By the end of the second hour most animals were easily aroused, but nevertheless exhibited severe ataxia in the hind limbs. Slight ataxia has been observed as late as 6 hours after injection.

Nembutal sedation during proestrus. In the exploratory experiments, 16 proestrous rats were each given a single injection of Nembutal at about 2 P.M. (table 1; cf. fig. 1, C). Seven of the 16 were killed on the morning after injection. In every instance ovulation had been prevented. The ampullae of the oviducts were not distended, nor were any tubal ova found. The ovaries contained from 8 to 10 large, growing follicles which gave no histological evidence of preovulatory swelling (fig. 6, plate II). The interstitial tissue remained markedly fatty, a further sign that LH release had been prevented (cf. Claesson and Hillarp, 1947; Everett, Sawyer and Markee, 1949; Everett, 1949, b). The uteri remained greatly distended and hyperemic. The other 7 rats were allowed to survive longer. Four of them were killed on the second morning after injection. In all 4 of them the ampullae were greatly distended, a fact which in itself is strong presumptive evidence of recent ovulation. Direct examination for tubal ova in 2 cases revealed 10 to 11 eggs; the examination was omitted in the other 2 rats. The uteri were no longer distended. The ovaries were hyperemic and contained fresh corpora lutea (fig. 7, plate II) in a stage of development such as would ordinarily have been found 24 hours earlier. The interstitial lipid was considerably depleted. The remaining 5 rats were killed on the third day after injection. Of these, 4 pairs of ovaries contained sets of recent corpora lutea (fig. 8, plate II), corresponding histologically with those which are normally found on the second day after ovulation. The fifth pair contained 14 lutein cysts of comparable age. Therefore, it was apparent that in all 14 animals the treatment with Nembutal had prevented the ovulatory discharge of LH during the day of proestrus.

Ten other proestrous rats were injected with Nembutal at about 4 P.M. (table 1). Seven of the 10 ovulated normally overnight (fig. 1, B), having from 8 to 10 tubal ova at the time of autopsy the next morning. Their corpora lutea were closely similar to those normally seen at that stage of the cycle (fig. 3, plate I). One other rat was ovulating at the time of autopsy and is therefore judged to represent partial interference with LH release. She had only 1 tubal ovum and 2 recognized rupture points in the corresponding ovary. The other ovary contained 4 follicles in preovulatory swelling and a luteinized cyst with retained ovum. The remaining 2 rats failed to ovulate or to give histological evidence of partial activation.

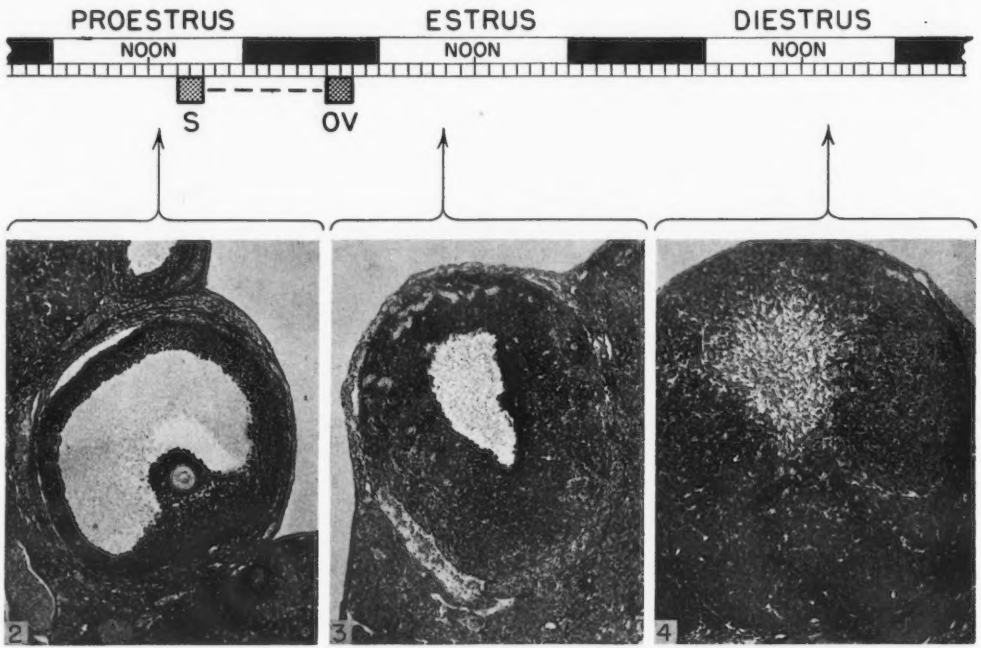

PLATE I. Follicle and corpus luteum histology characteristic of proestrus and the succeeding 2 days of the 4-day cycle, for comparison with experimental modifications depicted in plates I–IV. "S"—pituitary activation. "OV"—ovulation time. Hour of autopsy in each case is indicated by arrow. ×60.

These results compare favorably with those which followed the administration of other blocking agents at these hours (Everett, Sawyer and Markee, 1949).

As controls, 4 rats were each injected intraperitoneally with 0.1 ml. of 20% propylene glycol in 10% alcohol at 2 P.M. on the day of proestrus. By the following morning each rat had ovulated from 9 to 10 ova. No evidence of interference with LH release was found. Thus, it was demonstrated that neither the solvent nor the fact of intraperitoneal injection as such are factors in the blockade of the LH-release apparatus by Nembutal. This

TABLE 1. EFFECTS OF NEMBUTAL ON LH RELEASE

No. of rats	Nembutal dose (mg./kg.)	Injection times			Results[3]		
		Day 1 (proestrus)	Day 2	Day 3	Blocked	Partially blocked	Not blocked
4	30	10 A.M.			0	0	4
6	30	12 Noon			4	0	2
4	50	2 P.M.			4	0	0
12	30	2 P.M.			12	0	0
10	30–50	4 P.M.			2	1	7
cumulative 51[1]	30	2 P.M.			50 (98%)	1	0
6	30	2 P.M.	2, 4 P.M.		0	0	6
21[2]	30	2 P.M.	2, 4 P.M.		18 (86%)	2	1
3	30	2 P.M.	2 P.M.	2 P.M.	1	2	0
10	30	2 P.M.	2, 4 P.M.	2, 4 P.M.	9 (90%)	0	1

[1] Including the 27 cases listed as treated on day 2, and 12 cases receiving atropine on day 2 (see text).
[2] Including the 13 cases listed below.
[3] With particular reference to the effect within 24 hours after the last treatment indicated. Those blocked were, of course, also blocked by any earlier treatment.

was supported later by the demonstration that other barbiturates will readily block, although dissolved in physiological saline and injected subcutaneously (see below).

At this point, an estimate was required as to how long a single 30 mg./kg. intraperitoneal injection of Nembutal remains effective, a question which was easily tested by administration at hours earlier than 2 P.M. Of 6 proestrous rats injected at noon, 4 were blocked (table 1). Autopsy the next morning revealed the absence of tubal ova and the presence of full sets of unruptured follicles without evidence of preovulatory swelling. The other 2 rats had full sets of tubal ova at that time. Four rats which were injected at 10 A.M. during proestrus ovulated normally overnight, having from 8 to 10 tubal ova the next morning. In the 4 rats which were blocked by the noon injection it must be assumed that the Nembutal was effective for at

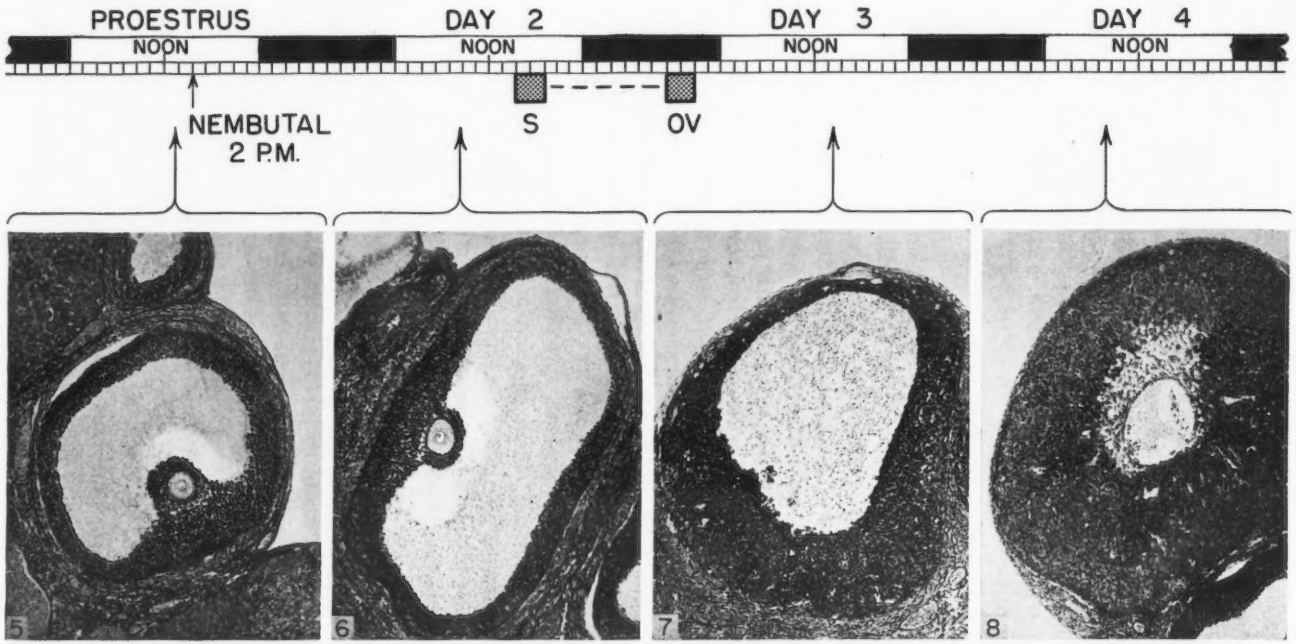

PLATE II. Follicle and corpus luteum histology showing characteristic results of blockade of pituitary activation by Nembutal during proestrus. Figure 5 duplicates figure 2 (plate I). In figure 6 note increased size of growing follicle, the compact, well organized granulosa and the intact ovocyte nucleus. In figure 7 the fresh corpus luteum is not significantly different from that in figure 3, when allowance is made for the difference in hours of autopsy. The same is true of the corpus luteum in figure 8 as compared with that in figure 4.

least 4 hours. In those which were injected at 10 A.M. the effect was evidently lost in less than 6 hours.

Demonstration of 24-hour periodicity in the LH-release apparatus. If, when Nembutal is injected at 2 P.M., it simply delays activation of the hypophysis for a few hours, we should have found signs of preovulatory swelling by 8 A.M. the next morning. Such was not the case, however, as indicated above. Inasmuch as evidence of a 24-hour rhythm in the LH-release apparatus had been encountered in the study of progesterone-advanced ovulation (Everett and Sawyer, 1949, b), it might reasonably be looked for here.

Exploring this possibility, 10 rats were first given Nembutal at 2 P.M. during proestrus. On the following day, 5 of them were given the standard blocking dose of atropine at 2 P.M., while the other 5 were given atropine at 4 P.M. All of the former were blocked by the atropine, while the latter ovulated during the following night (the second night of the experiment). Of the former group, 3 rats were autopsied on the morning after the atropine treatment, at which time their ovaries contained full sets of large, growing follicles without evidence of preovulatory swelling. The remaining 2 rats of the 2 o'clock-atropine group were continued an additional day. Their ovaries contained at that time 9 to 10 large follicles in early atresia. In the 4 o'clock-atropine group, all animals were killed on the morning after the atropine injections. Each one possessed from 6 to 9 tubal ova and the ovaries contained fresh rupture points indicative of ovulation a few hours earlier.

It was, therefore, clearly evident that once blockade is produced with Nembutal during the critical hours on the day of proestrus, activity of the LH-release apparatus is postponed until similar hours the next day. This delay is not a direct effect of Nembutal *per se*, for we have shown that its specific inhibitory action lasts only a few hours. The 24-hour periodicity must then be a property of the neural component of the LH-release mechanism.

Nembutal sedation on successive days. In the light of the above experiments, an attempt was made to block pituitary activation for 2 days in succession by giving Nembutal at 2 P.M. each day, beginning during proestrus (fig. 1, D). Six rats were treated in this way and, surprisingly enough, all of them ovulated during the second night (table 1). Therefore, on the second day pituitary activation must have occurred at nearly the normal time in spite of the Nembutal. Two additional rats, however, treated exactly as we had treated the above 6 animals, were further given the standard blocking dose of atropine at 4 P.M. on the second day. These were blocked: at autopsy the next morning neither animal had tubal ova; the 2 pairs of ovaries contained 9 to 11 large follicles. This finding indicated

that failure of the single injection of Nembutal on the second day results either from a shortened action of the drug due to acquired tolerance or from a more prolonged period of excitability of the LH-release apparatus. Whatever the explanation, the next step was simply to inject Nembutal at both 2 P.M. and 4 P.M. on the second day.

In 21 rats, each of which had received the single blocking dose of Nembutal at 2 P.M. during proestrus, the treatment was repeated the following afternoon at 2 o'clock and again 2 hours later (fig. 1, E; plate III). Included in this group are 13 rats which were given additional Nembutal on day 3 (table 1) and in which the histological findings show that blockade had been uniformly successful on the first and second days. Among the other 8 rats, 5 were blocked (figs. 11 and 12, plate III) and 2 were partially blocked by the double injection on day 2. The remaining one ovulated the second night in spite of the treatment.

Among 10 rats treated for 3 days in succession, with the single injection during proestrus and double injections on the next 2 days (table 1), 9 were blocked throughout (fig. 1, F; plate IV), while 1 evidently ovulated during the third night in spite of the double injection that afternoon. She was killed on day 5. Her ovaries contained a full set of corpora lutea, corresponding histologically with those which are normally found about 30 hours after ovulation (cf. figs. 4 and 8). Of the 9 which were blocked, 6 were killed on the morning of day 4, while 3 were allowed to survive an additional day without further treatment. The ovaries of all 9 rats contained full sets of large follicles. In the serial sections, none of these gave any indication of luteinization or preovulatory changes. They all showed evidence of early atresia, especially in the parietal granulosa (fig. 16, plate IV): absence of mitotic figures, local or widespread disorientation and thinning, and occurrence of densely staining intercellular granulations. No evidence of deterioration of the ova was recognized; the nuclear membranes were intact.

In addition to the 10 rats just mentioned, 3 others which had been similarly treated on the first 2 days were each given a single injection of Nembutal on the third day at 2 P.M. (table 1). At autopsy the next morning 1 rat had 10 large follicles, later found to be atretic. Another had 3 (+2?) follicles in preovulatory swelling and at least 1 follicle which had luteinized without rupturing. The third rat had 3 tubal ova, corresponding rupture points and 6 luteinized, unruptured follicles. The latter 2 cases are judged to represent partial blockade only. The double injection is thus desirable on the third day as well as on the second.

Recapitulation of Nembutal results. Reviewing the experiments as outlined in figure 1, it appears that the selective blocking-out of the LH-release mechanism by daily Nembutal treatment discloses an underlying

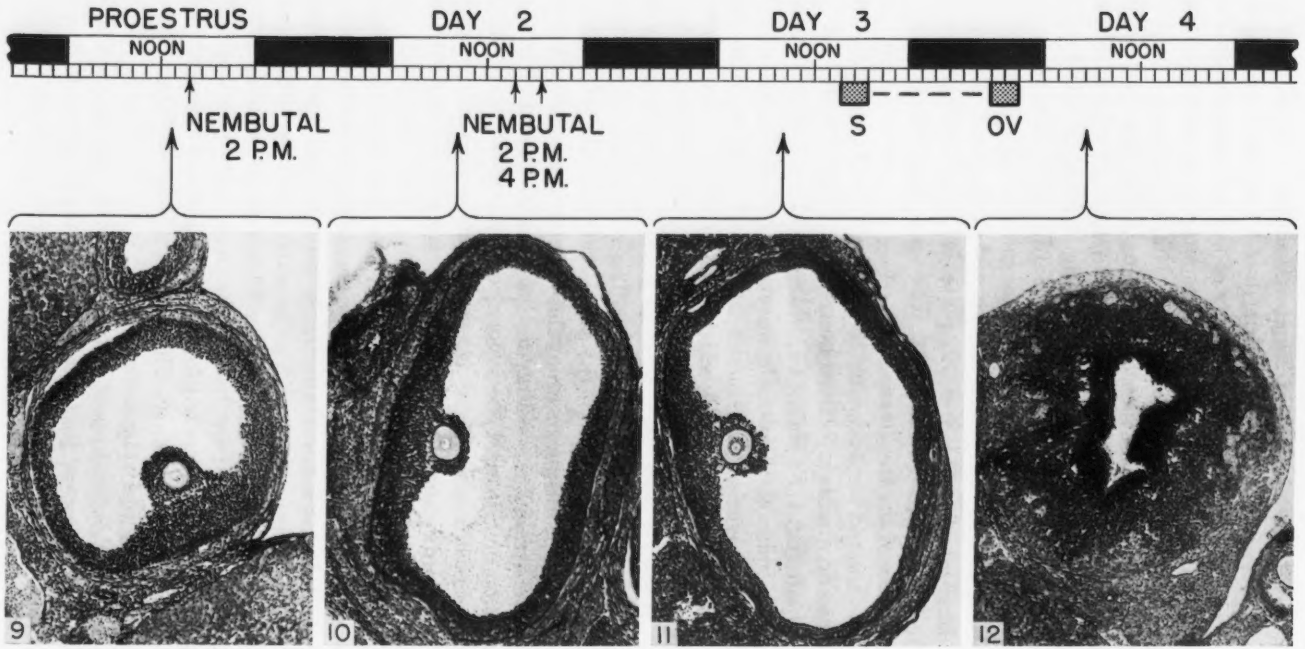

PLATE III. Results of blockade of pituitary activation for 2 successive days. Figures 9 and 10 duplicate figures 5 and 6 (plate II). In figure 11 note that little growth of this follicle is evident when compared with figure 10. The granulosa is somewhat thinner, but remains well organized. The fresh corpus luteum in figure 12 is practically identical with that in figure 3 (plate I).

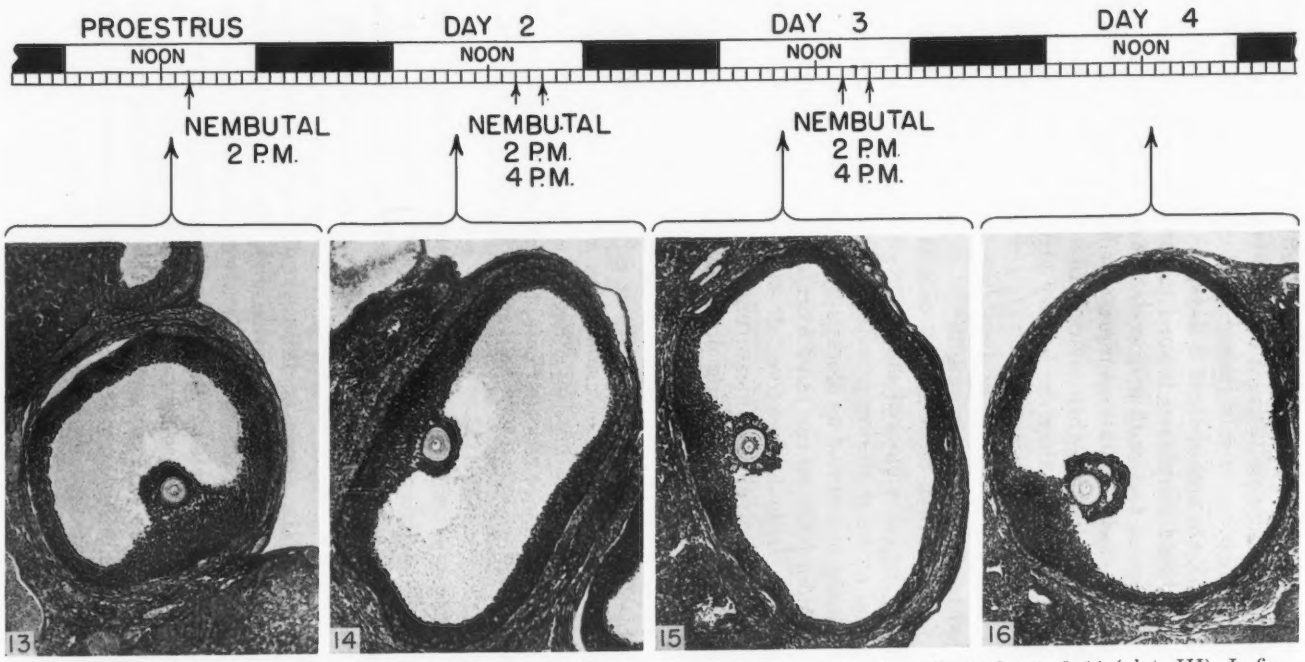

PLATE IV. Results of blockade of pituitary activation on 3 successive days. Figures 13–15 duplicate figures 9–11 (plate III). In figure 16 note the marked thinning of the parietal granulosa. Although the ovum shows little change, this follicle is atretic.

follicular cycle (fig. 1, F, and plate IV), which is on the upgrade during proestrus, at a maximum on the next day and beginning to regress on day 3. (There is no great increase in follicle size between day 2 and day 3—figures 14 and 15). On any of these 3 days, if the appropriate Nembutal treatment is withheld or delayed beyond the critical hours, activation of the hypophysis occurs forthwith and ovulation follows during that night. By the third day, under conditions represented in figure 1, E and F, the follicles have so nearly "run their course" that unless LH release occurs that afternoon (plate III) they are frankly atretic by the next morning.

The nature of the experiments involving treatment with Nembutal or atropine on day 2 or with Nembutal on days 2 and 3, is such that they may be added to the data pertaining to the day of proestrus. Obviously, an animal which had been blocked for 2 or 3 days in succession should be counted among the cases blocked during proestrus. Similarly, an animal given Nembutal at 2 P.M. during proestrus and atropine at 4 P.M. on the second day, should be counted as blocked by the Nembutal, for the freshness of the corpora lutea on the third morning excludes ovulation during the first night. In table 1, therefore, all such experiments are included among the 51 (cumulative) cases measuring the effectiveness of 30 mg./kg. Nembutal injected at 2 P.M. during proestrus. In the 1 exceptional case listed as "partially blocked" a solitary follicle, although unruptured, was luteinized during the night following proestrus.

While double injection at 2 P.M. and 4 P.M. on the second and third days was generally successful, the occasional failures indicate that in some animals the effect of the 2 P.M. injection wore off in less than 2 hours. In more recent studies, not reported here, the interval has been shortened to 1½ hours. No failures have been encountered among 16 cases injected at 2 P.M. and 3:30 P.M. on day 2.

Blocking action of other barbiturates. Amytal, dial, barbital and phenobarbital are fully as effective as Nembutal in blocking the LH-release apparatus (table 2). Of especial interest are the experiments with the 100 mg./kg. dose of barbital and those with phenobarbital, for here the outward effect is merely a slightly ataxic gait. With the 50 mg./kg. dose of phenobarbital, even the ataxia is difficult to recognize. A treated rat, caged with untreated animals, can usually be distinguished only after close scrutiny. In spite of this minimal outward effect, the 50 mg./kg. dose of phenobarbital was almost uniformly effective in blocking the LH-release mechanism during proestrus. It was, however, considerably less effective when repeated on the second day (table 2), offering a suggestive similarity to the diminished effectiveness of Nembutal on day 2. The apparent increased blocking capacity of phenobarbital on day 3 (table 2) may

not be real. The 7 rats included in this category were necessarily animals which had been blocked on the first 2 days. Such automatic selection would favor the more susceptible individuals.

Modified vaginal smear sequences. In previously reported work with other blocking agents, we often found that a significant retardation of the vaginal smear sequence took place during the night immediately following blockade (Everett, Sawyer and Markee, 1949). It was no surprise, therefore, to find similar results in the Nembutal series. Of 55 rats receiving

TABLE 2. BLOCKADE OF THE LH-RELEASE MECHANISM BY VARIOUS BARBITURATES

No. of rats	Agent	Amount (mg./kg.) and route of injection	Injection times			Results[2]		
			Day 1 (proestrus)	Day 2	Day 3	Blocked	Partially blocked	Not blocked
3	Amytal	100 s.c.	1:30 P.M.			3		
3	Dial	60 s.c.	1:30 P.M.			3		
2	Barbital	200 i.p.	12:45 P.M.			2		
4	Barbital	150 i.p. 200 i.p.	1:00 P.M.	1:00 P.M.		4		
4	Barbital	150 i.p.	12:45 P.M.			4		
2	Barbital	100 i.p.	1:15 P.M.			2		
3	Phenobarbital	75 s.c.	1:15 P.M.	1:15 P.M.	1:15 P.M.	3		
24[1]	Phenobarbital	50 s.c.	12:30–1:15 P.M.			22 (92%)	1	1
21[1]	Phenobarbital	50 s.c.	12:30–1:15 P.M.	12:15–1:30 P.M.		12 (57%)	1	8
7	Phenobarbital	50 s.c.	12:30–1:15 P.M.	12:15–1:30 P.M.	12:15–1:30 P.M.	6 (86%)	0	1

[1] Including all cases listed below.
[2] With particular reference to the effect within 24 hours after the last treatment indicated. Those blocked were, of course, also blocked by any earlier treatment.

Nembutal at 2 P.M. during proestrus, the vaginal smears of 23 animals (42%) were in late proestrus on the following morning.

In the experiments of longer duration, whether or not subsequent treatment was given, the vagina was always cornified on the second morning after the proestrus injection. Occasionally, a few nucleated cells were present among the cornified cells. By reference to figure 1, it may be seen that blockade for 2 or even 3 days may result in no greater prolongation of the cornified stages than blockade for only one day. In about half of these cases of repeated blockade the smears were strongly leucocytic on the third morning after the proestrus injection. In the others, we usually found a high proportion of cornified cells with a variable admixture of nucleated epithelial cells and occasionally a few leucocytes. Thus, while we have the means to determine whether a current set of follicles will ovulate on

the first, second or third nights following proestrus (or not at all), vaginal cornification can be prolonged little more than one day by any of these procedures.

Effects on the uterine cycle. Quite directly pertinent to the above mentioned dissociation between ovulation time and stages of the vaginal cycle, are the findings with respect to the uterus. When the LH-release mechanism was blocked with Nembutal for only 1 day, the uterus was consistently in the distended proestrous condition the next morning. However, when blockade was accomplished for 2 days in succession, uterine distention was never found on the third morning. This, coupled with the evidence from the vaginal smears, suggests that in daily blockade with Nembutal, estrogen secretion continued at maximal level for only about one extra day under any but the most exceptional circumstances.

Recurrence of cycles after Nembutal blockade. In a few instances, in addition to the cases reported in the definitive Nembutal series, rats were kept alive for several days or weeks after presumptive Nembutal blockade. Eighteen rats were each given Nembutal (30 mg./kg.) at 2 P.M. during proestrus. Five of them were further treated with the double injections (2, 4 P.M.) on the next 2 days. Among the 13 rats receiving only the 1 injection the onset of the next expected vaginal proestrus was delayed 1 day in 5 rats and 2 days in 8 rats. Among the 5 rats which were treated for 3 successive days the onset of the next vaginal proestrus was delayed only 1 day in each instance. Thus the new cycle was retarded no longer by blockade for 3 days than by blockade for only 1 day. Subsequent cycles usually recurred at 4-day intervals thereafter, although 3 rats (single injection group) had 5-day cycles for awhile and 1 became anestrous (pseudopregnant?) for about 2 weeks before renewal of 4-day cycles.

DISCUSSION

In retrospect, diurnal rhythmicity of the neural component of the LH-release apparatus in rats was implied in the initial demonstration that the ovulatory activation of the hypophysis in the 4-day cycle regularly occurs during proestrus between 2 and 4 P.M. (Everett, Sawyer and Markee, 1949). It was more apparent in the study of advancement of ovulation by progesterone in the 5-day cycle, where a limited period of sensitivity to progesterone occurs 24 hours before the normal time of pituitary activation (Everett and Sawyer, 1949, b). The present results confirm its existence by an entirely different method.

We have postulated (Everett and Sawyer, *loc. cit.*; Everett, 1950) the location of a diurnally excitable center in the rostral hypothalamus, a location suggested by the experiments of Dey *et al.* (1940, 1943) in guinea pigs and of Hillarp (1949) in rats. In both of these studies constant estrus

resulted from bilateral destruction of certain portions of the rostral hypothalamus. Hillarp's effective lesions were either diffuse ones in the anterior hypothalamic area adjacent to the paraventricular nuclei or narrowly restricted ones between that region and the median eminence. Estrogen and progesterone, in evoking LH release, may operate either by sensitizing the diurnally rhythmic center itself or by lowering thresholds more distally in the linkage between this center and the adenohypophysis.

It appears that this diurnally rhythmic component constitutes the means by which the estrous cycle is most precisely synchronized with environmental rhythms. Yet we are far from explaining the long-term effects of seasonal changes such as day length. The information presently at hand, however, will be a valuable point of departure in study of these more obscure phenomena.

There is an important similarity between the result of daily blockade with Nembutal and the state of persistent follicle produced in rats by continuous illumination (Browman, 1937; Hemmingsen and Krarup, 1937) or occurring "spontaneously" with advancing age (Everett, 1939, 1942). With respect to the ovary, the only marked difference seems to be that after daily Nembutal blockade the current follicles become atretic and inactive before a new set has gained competence, whereas in the other conditions the follicle generations overlap. The overlap is the result, not of earlier replacement by new follicles, but of longer retention of older follicles with healthy granulosa (Everett, unpublished). Furthermore, the older follicles often attain larger volume than those we have seen in the Nembutal experiments. Fiske (1941) reported that continuous illumination of female rats results in a significant increase in FSH secretion and diminished LH, when estimated by the augmentation reaction. Because of close similarities of ovarian histology in "light estrus" and in "spontaneous persistent estrus," a similar shift in hormone balance could be postulated in the latter condition, thus accounting for the longer, overlapping follicular cycles in either instance. From this point of view, the shorter follicular cycles in the Nembutal experiments would represent no abnormality in hormone output, aside from the specific blocking-out of the ovulatory discharge of LH. There remain, nevertheless, the alternative possibilities of interference by Nembutal with trophic action of the hypothalamus on the hypophysis or of non-specific interference with gonadotrophin production through metabolic disturbances. The first alternative is favored by the fact that the onset of the next proestrus is delayed no longer by 3-day treatment with Nembutal than by the single injection.

The fact that ovulation is held in abeyance by continuous light much as it is by daily blockade with barbiturates, implies that this function of light is elicited by inhibition of the diurnal excitability of the LH-release

apparatus. There is evidence at hand to suggest in part how this may be accomplished. The effect is wanting in blinded or congenitally blind rats (Browman, 1937–1943). There are said to be direct nerve fiber connections from the retina into the hypothalamus, in particular to the supraoptic nuclei and tuber cinereum (cf. Marburg, 1942). Other indirect connections may, of course, be equally important. From the above cited studies by Dey *et al.* and by Hillarp, it appears that destruction of certain regions in the rostral hypothalamus has an effect similar to that produced (in rats) by continuous light: constant estrus.

It would be valuable to know whether Hillarp's rats retained their diurnal activity rhythms. One investigator (Nauta, 1946) has reported constant wakefulness in rats following transection of the rostral hypothalamus. In continuous illumination of normal rats, on the other hand, an approximate 24-hour activity rhythm is retained (Browman, 1937; Hemmingsen and Krarup, 1937), even when room temperature remains constant (Browman, 1943). However, the rhythm apparently loses its former close association with the solar day-night sequence.

Although activity, psychic estrus and LH release are somewhat similarly conditioned by the sex steroids and, in rats, are all synchronized with the diurnal lighting rhythm, their interrelationships are obscure. For example, in our 4-day cyclic rats, during the critical hours of 2 to 4 P.M. during proestrus, the animals are quiescent and usually sleeping unless disturbed. The onset of estrous behavior is characteristically later than 7 P.M., as measured by acceptance of the male. After Nembutal blockade during proestrus about two-thirds of the rats come into heat that night, in spite of the failure of LH release (unpublished). Usually they are out of heat by 8 A.M. and in heat once more by mid-afternoon.[2] On this second day, therefore, the onset of heat approximates the time of pituitary activation, whereas during normal proestrus several hours normally intervene between the two events. The difference may be an expression of modified thresholds. Experiments to be published elsewhere suggest that the greater difficulty in blocking pituitary activation on the second day of the barbiturate experiments results from more porlonged action of (or increased amount of) estrogen, together with increased progesterone. It is known that these hormones also act synergistically in the induction of psychic estrus. Thus it appears that while pituitary activation may occur near the onset of heat if progesterone-estrogen synergy is maximal, under other circumstances the former process may take place "silently" several hours in advance of the latter. From this it follows that the interval between onset

[2] The term "split estrus" was coined by Young *et al.* (1935) to describe a similar, naturally occurring, intermittent estrus in guinea pigs. Blandau *et al.* (1941) reported that split estrus sometimes occurs in rats under ordinary laboratory conditions.

of psychic estrus and ovulation may be considerably more variable than the interval between pituitary activation and ovulation. It is common knowledge, in fact, that ovulation may sometimes occur "silently" in the absence of overt sexual behavior.

Effects of barbiturate sedation over periods corresponding to more than one follicular cycle were not studied in the present investigation. Westman (1947), however, treated 21 adult female rats with twice-daily injections of Prominal (1-methyl-5, 5-ethylphenyl barbituric acid) for 18 to 20 days. The initial treatments were apparently given at random stages of the cycles. Three rats became anestrous, 6 became "irregular," while 5 continued to have "normal" cycles. Of especial interest, from our standpoint, is the fact that 7 rats exhibited constant estrus. Unfortunately, although 4 of the 7 were killed during the experiment, no histological study of the ovaries is reported. It is merely stated that the ovaries were enlarged and contained "swollen" follicles, without mention of presence or absence of corpora lutea.

To our knowledge, there is no other record in the literature to indicate that central depressants block the ovulatory discharge of gonadotrophin in spontaneously ovulating animals. The present results suggest, however, that such an effect may be demonstrable, not only in rats, but in other species as well. In the exploration of this question the possible pitfalls are many. For example a diurnal rhythm of excitability of the LH-release apparatus may exist in only certain species, while in others the neural neural mechanism may be continuously near threshold for perhaps several days during each cycle. If periodic excitability is actually the rule. It may in some species be demonstrable under only the most rigid control of (physical and social) environmental conditions, the periods being otherwise erratic.

There is no *a priori* reason to assume that in all spontaneously ovulating animals the specific ovulating stimulus to the hypophysis is as sharply limited in time as in the rat. Unpublished data indicate that in this species such activation of the anterior lobe takes place over a period somewhat longer than 15 minutes, considerably longer than in the rabbit, where activation occurs within one minute after copulation (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949). Conceivably, there are species in which "spontaneous" activation may extend for a much longer time than in rats.

Other factors to be considered in the study of these questions have to do with duration of action and complicating side effects of any drug which may be employed. Had we not had advance knowledge of the critical hours for pituitary activation in rats, the present investigation might well have yielded results no more illuminating than those of Westman cited

above. Nembutal, chosen quite fortuitously, proved to be ideally suited to the species with which we worked. The onset of action after intraperitoneal injection is sufficiently rapid and the duration of its suppressing effect on pituitary activation is just long enough to allow definition of the time limits of activation as accurately as was possible with atropine. For an hypothetical species having a more prolonged activation stimulus than that in rats, the use of a long-acting agent or repeated administration of one having medium or short action would be necessary. Continuous excitability over several days would require continuous sedation. Here one would encounter other problems.

There seems to be no direct relationship between the extent of depression of locomotor function, reflexes, etc., on the one hand, and inhibition of the LH-release mechanism on the other. In the Nembutal series a few rats failed to be blocked on the second or third day even by the double injection procedure, indicating that pituitary activation took place shortly before the second injection (4 P.M.). Yet these animals were still markedly ataxic at that time. In the phenobarbital series, however, effective blockade of LH release was accomplished with doses whose outward manifestation was limited to a barely perceptible impairment of gait. Thus while the latter drug can block LH release with only slight suppression of other neural functions, relatively deep narcosis with Nembutal may be followed by pituitary activation during the recovery phase while the external signs of depression are still marked.[3]

Because of the above considerations, it seems to us that experimental treatment with the barbiturates or other depressant drugs over a period of days at the height of long follicular cycles (in primates, for instance) would give significant information only in the event that the following results were realized: (1) failure of ovulation and luteinization throughout the period of treatment; (2) ovulation or, at least, luteinization within reasonably constant time limits after withdrawal of the drug, whenever that might be.

SUMMARY

Earlier investigation by the use of Dibenamine and atropine indicated that in our 4-day cyclic rats a specific neurogenic stimulus, essential for the ovulatory discharge of LH, activates the hypophysis between 2 and 4 P.M. on the day of proestrus. Evidence of a 24-hour rhythm of excitability in the neural component of the LH-release apparatus was also recently reported. The present results confirm the earlier ones by different methods.

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[3] A similar escape of the LH-release apparatus probably occurred in an experiment reported by Nicholas and Carmosino (1944). This would account for the ovulation which apparently took place during prolonged amytal narcosis.

It is shown that Nembutal will prevent the ovulatory activation of the hypophysis when administered at 2 P.M. during proestrus. Persistence of graafian follicles for 2 to 3 days results when Nembutal treatment is repeated on successive afternoons at the *same critical hour*. On the second and third days a supplementary injection is required at 3:30 to 4 P.M. If, on any of these 3 days treatment is omitted or postponed until after 4 P.M., pituitary activation will occur promptly and ovulation will follow during that night.

Similar results are produced by amytal, dial, barbital and phenobarbital.

Blockade of LH release by treatment with the barbiturates on successive days discloses a follicular cycle which terminates in atresia about 2 days before onset of the next proestrus. Consideration is given to similarities and differences between such follicular cycles and the overlapping ones found in "spontaneous persistent estrus" and "light estrus."

The 24-hour rhythm, although possibly a property of the LH-release mechanism in other spontaneously ovulating forms as well as the rat, cannot be considered as a universal characteristic until a variety of animals has been investigated.

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